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LETTER FROM THE EDITORS

Dr. Leon O. Jacobson and Dr. Oliver H. Lowry have completed, respectively, 6- and 2-year terms on the Board of Editors. On behalf of the Central Society for Clinical Research and the Editorial Board, we wish to express our appreciation for their valuable services.

Dr. Richard L. Landau and Dr. Oscar D. Ratnoff have accepted appointments on the Board of Editors.

During the past year the Editors and Board members have called upon many of their associates and colleagues in other universities to aid them in the evaluation of manuscripts. Those who have participated in this capacity in the past year are:

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We wish to take this opportunity to thank the past and present members of the Editorial Board for their excellent services to the JOURNAL and the Central Society for Clinical Research. We would also like to express our appreciation for the cooperation received from The C. V. Mosby Company. We particularly wish to thank the retiring Editors, Dr. Clayton G. Loosli and Dr. Robert H. Ebert, for their invaluable help and advice during the past year.

WILLIAM D. ROBINSON, M.D.
Editor
WILLIAM H. BEIERWALTES, M.D.
Associate Editor

MEDICAL EDUCATION AND TRAINING IN RESEARCH

CLAYTON G. LOOSLI, M.D.

MEMBERS and guests of the Central Society: What I have to say today can be placed most appropriately under the category of remarks. It has been a high honor for me to serve this Society as President. Our efficient Secretary, Dr. Ebert, has made my task an easy one. I have enjoyed the association and appreciated the help of the Council in the selection of new members and of the papers you will hear today and tomorrow.

Since 1948, as Editor of the Journal or an officer, I have attended the annual Council meeting when candidates for membership in the Society were considered. I assure you that this responsibility has been accepted with utmost seriousness and objectivity by the officers and Council members. For active membership in this Society a candidate must be a practicing physician who has "acomplished a meritorious original investigation in the clinical or allied sciences of medicine." If this statement had been interpreted literally by the past and present Councils few would have qualified, because the word "original" would have eliminated most of us. Be that as it may, however, supporting evidence of the candidate's qualifications includes a summary of his academic training, his present academic position, letters of recommendation from two or more members of the Society, a list of published scientific reports, and evidence of moral integrity. All of these factors are weighed carefully by the Council, but moral integrity and the bibliography, which supplies the evidence of "meritorious original investigation," are the most important considerations.

If a candidate is not elected to this Society the first year, he has two more years during which to qualify. Only a few fail to be elected after three years. The reasons are varied. A few candidates leave the area of eligible membership. Some discontinue their researches and enter private practice. Others after making a creditable start as investigators show no further progress during their next two years. It is this group about which the Council spends many hours. Most of these individuals are earnest and qualified young men who are overburdened with administrative, teaching, or private practice responsibilities to the extent that their research programs bog down. I recall the application of one candidate for membership in our Society who was obviously striving to carry on an acceptable research program along with many other duties listed by his sponsors. His bibliography consisted of two published papers and interesting titles of seven others to be published. In support of his

Presidential Address given at the Twenty-eighth Annual Meeting of the Central Society for Clinical Research, Chicago, Ill., Nov. 4, 1955.

From the University of Chicago School of Medicine, Chicago, Ill.

application the third year, his sponsor submitted a list of five additional papers to be published, with no comment concerning the whereabouts of the seven manuscripts which were "to be published" two years previously. I submit again that if those responsible for the training of this young investigator had the true spirit of our Society, namely "the cultivation of clinical research and the development of younger men," they would have relieved him of some of his teaching responsibilities so that he might have had time to prepare his possibly "meritorious and original investigations" for publication.

Thus, the failure of some of these worthy individuals to be elected to our Society rests on the shoulders of the chiefs of their respective departments. In many of our medical schools, time during which a young man is expected to do investigative work and report the results is not infrequently given a low priority by his "Chief." Research programs which must be performed before 9 A.M. and after 5 P.M. will not attract many individuals, even those of good intelligence, internal drive, willingness to work hard, and tenacity of purpose, into a career of scientific endeavor. Having time to think, plan, and execute experiments, read the literature, correlate the findings, and report the results is essential in the training of the young investigator if we are to develop the "inquiring mind,"[1] which is so fundamental in the art of scientific investigation.[2, 3]

Beveridge states that "curiosity and love of science are the most important mental requirements for research" and that "perhaps the main incentive is the desire to win esteem of one's associates, and the chief reward is the thrill of discovery. . . ."[2] As medical school faculty, we have a responsibility for teaching both the art of medical practice and the art of scientific investigation. The latter can develop and flourish in young men only in friendly, sympathetic, and stimulating environments[3, 4]—environments in which their superiors have, or are feeling, the "spirit of pure delight" from their own accomplishments in the field of medical research.[5]

Teaching only what is known in medicine is a hollow goal and cannot be considered a scholarly endeavor. Training in wisdom and knowledge of the technical aspects of medical practice is not enough.[6, 7] Scientific discipline in medical practice is not antagonistic to human kindliness.[8] Bertrand Russell has stated that most of his opinions spring ultimately from admiration of two qualities: kindly feeling and veracity. Dr. Platt, Professor of Medicine at the University of Manchester, saw in these qualities "the art and science of medicine." The art was "the wish to help and above all to understand the human being in distress." The science was "the discipline to withstand all temptation to depart from accuracy and the determination to advance knowledge by the methods of science which are ultimately concerned only with the search for the truth."[6] This, I believe, should be our goal in medical teaching today.

I would like to comment on another problem which I believe should concern all the members of this Society. It is the constantly decreasing supply of qualified teachers and investigators in the clinical and allied basic sciences. In the annual (1953-54) report of the Educational Testing Service,[9] it is

stated that "less than half of those equal in ability to the average college graduate now enter college. Even among the top two per cent of our high school graduates, only two-thirds are now going all the way through college. Whereas six or more per cent of the population are capable of acquiring a Ph.D. or an M.D., the number earning these degrees at present is less than half of one per cent of the population." Dr. Rappleye,[10] in discussing the problem of maintaining standards of medical education, states that "our nation must depend upon the brain power of its scientists, physicians, and research workers to continue the leadership of the world." Our medical schools will occupy their proper place in this leadership only if they continue to recognize their responsibility to train and develop investigators as well as practicing physicians.

Many of you are aware of the fact that since 1947 there has been a steady decline in the number of applicants seeking admission to medical schools. In 1954-55 there were approximately 14,500 applicants for approximately 7,500 freshmen places, or a ratio of applicant to place of only about 1.93.[11] With the decline in the number of applicants there has been also, as would be expected, a decline in the number of highly qualified individuals who are sought after by all medical schools. It has been pointed out by Dr. Stalnaker that some of the "schools with geographic restrictions had to scrape 'the bottom of the barrel' to get a freshman class this year, and no school had as many topflight applicants as it desired." In the past, many of those not gaining admission to medical school sought training in one of the allied sciences, where they went on to distinguish themselves in teaching and investigation. In addition, many of the present leaders in the basic medical sciences were lured from the ranks of the medical graduate. This is not so today. Although we might like to place the blame of the lack of qualified investigators in the medical sciences on the doctors' draft, this factor is probably not very important. One important factor, of course, is the high cost of a career in medical teaching and research, with an investment of from eight to twelve years after high school, for which there is only moderate compensation in salary after the period of training is completed.

In June, 1955, approximately 7,000 students were graduated from the American medical schools, having achieved this end through a maze of medical curricula with emphasis on medical care. In the introduction of the annual report of the Council on Medical Education and Hospitals, it is stated that the graduate should "possess a reasonable understanding of science, man, his health, diseases, and disabilities. He should have developed a genuine spirit of curiosity and be in the possession of methods of study which foster the accumulation of facts and the making of observations that lead to new knowledge and the wisdom to utilize it."[12] However enthusiastic we might be about our respective undergraduate training programs, they fall far short of this goal. The graduating medical student, because of the crowded and lockstep curriculum, learns little of the experimental method and principles of the basic sciences so essential to the comprehension of clinical medicine and the

care and supervision of the sick. Thus there is the necessity for postgraduate training. This starts with the internship. Immediately thereafter the ax of specialization begins the process of the splintering away of whatever broad concepts of medicine might have been acquired as an undergraduate, so that little is left at the end of the three- to five-year postgraduate training period.

As of June, 1955, there were 11,000 internships for our 7,000 graduates. Approximately 4,800 of these are in medical school affiliated hospitals, while over 6,000 are in unaffiliated hospitals. In spite of the added inducement of better training in the science and art of medicine, only forty-six per cent of the graduates last year interned in university supervised hospitals. The number of residency places for postgraduate training is approximately 25,400, and only about 26 per cent are under medical school control.[13] The opportunities for better living facilities, stipends, and other material benefits for interns and residents in the nonaffiliated hospitals are such that many of the better students also accept appointments in these institutions where little or no basic scientific teaching or research is carried on. Other highly qualified individuals give way to economic pressure and enter private practice. This and the loss of qualified individuals to the nonaffiliated hospitals are resulting in a decreasing number of intellectually desirable graduates for advanced training in academic medicine at the university centers.

This is a most serious problem. Stress in medical education today, both in the undergraduate and postgraduate years, is essentially on training for the practice of medicine.[14, 15, 16] Training in the art of scientific investigation, which is so essential in the development of teachers of medicine, is given little formal attention by the professional medical educators. More planned effort should be made both at the undergraduate and graduate levels to direct and train those most intellectually and tempermentally qualified for careers in academic medicine. The quality of medicine of the future depends on the caliber of the young men we secure today for advanced training to be teachers of medicine.[17] The medical school functioning within the framework of a university, as Dean Dorst has put it,[18] where the spirit of scholarly inquiry is ever present, should serve as the continuing source of future teachers and researchers in both the basic and clinical sciences. Advancement of medical knowledge and of the art of medicine depends on a faculty continually vitalized by younger colleagues in the pursuit of the unknown in the vast area of medical empiricism. High standards of intellect and scholarship among the medical faculties must be maintained if medicine is to remain a profession and medical centers "fountains of learning."

REFERENCES

1. Spink, Wesley W.: The Inquiring Physician, J. Lab. & Clin. Med. **37**: 2, 1951.
2. Beveridge, W. I. B.: The Art of Scientific Investigation, New York, 1949, W. W. Norton and Co., Inc., p. 154.
3. Cannon, W. B.: The Way of An Investigator, New York, 1945, W. W. Norton and Co., Inc.
4. Stead, Eugene A., Jr.: Presidential Address, 45th Annual Meeting American Society for Clinical Investigation, J. Clin. Invest. **32**: 548-549, 1953.
5. Allen, E. V.: The Pure Delight, J. Lab. & Clin. Med. **35**: 2, 1950.

6. Platt, R.: Wisdom Is Not Enough, The Lancet **2**: 977, 1952.
7. Mallick, S. M. K.: Medicine—a Technology or a Profession. Proceedings First World Conference on Medical Education, London, 1953, Oxford University Press, p. 29.
8. Arnott, W. Melville: The Aims of the Medical Curriculum. Proceedings First World Conference on Medical Education, London, 1953, Oxford University Press, p. 278.
9. Chauncey, H.: Annual Report to the Board of Trustees, 1953-54, Educational Testing Service, Princeton, New Jersey.
10. Rappleye, W. C.: The Struggle to Maintain Standards, The Pharos (Alpha Omega Alpha Society Publication) **17**: 3, 1953.
11. Stalnaker, J. M.: The Study of Applicants 1954-55, J. M. Educ. **30**: 625, 1955.
12. Turner, E. L., Wiggins, W. S., and Tipner, A.: Medical Education in the United States and Canada. Annual Report, Council on Medical Education and Hospitals, J. A. M. A. **159**: 563, 1955.
13. Leveroos, E. H., Springall, A. N., and Heinze, C. T.: Annual Report on Internships and Residencies. Council on Medical Education and Hospitals, J. A. M. A. **159**: 251, 1955.
14. Deitrick, J. E., and Berson, R. C.: Medical Schools in the United States at Mid-Century, New York, 1953, McGraw-Hill Book Co., Inc.
15. Report, First Teaching Institute, The Teaching of Physiology, Biochemistry and Pharmacology, Assoc. Amer. Med. Colleges, J. M. Educ. **29**: Part 2, 1954.
16. Report, Second Teaching Institute. The Teaching of Pathology, Microbiology, Immunology, Genetics. Assoc. American Medical Colleges, J. M. Educ. **30**: Part 2, 1955.
17. Wood, W. Barry, Jr.: Teachers of Medicine, J. LAB. & CLIN. MED. **41**: 6, 1953.
18. Dorst, S. E.: The Medical School Within the "Idea of a University," J. M. Educ. **29**: 11, 1954.

CLINICAL AND EXPERIMENTAL

THE PRESERVATION OF POLIOMYELITIS VACCINE WITH STABILIZED MERTHIOLATE

EDWIN O. DAVISSON, PH.D., HORACE M. POWELL, SC.D., JOHN O. MACFARLANE, PH.D., ROLAND HODGSON, PH.D., ROBERT L. STONE, M.S., AND CLYDE G. CULBERTSON, M.D.
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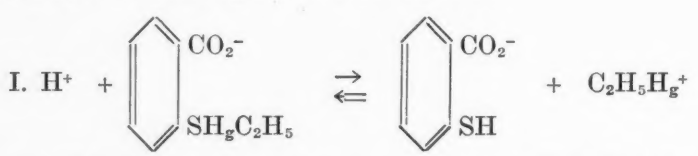
INTRODUCTION

MERTHIOLATE* was first used as a biologic preservative in 1931.[1] Since that time, this antiseptic has been used for this purpose in a wide range of products including vaccines, toxoids, antigens of one kind or another, plasma, antisera, antitoxins, and others. Some of the properties of Merthiolate which contributed to its acceptance as a preservative in parenteral preparations are (1) water solubility, (2) compatibility with biologic materials, (3) relatively low toxicity, and (4) effectiveness as a growth inhibitor of bacteria and fungi.

Our present interest in re-examining the use of Merthiolate as a biologic preservative was stimulated by information from Dr. Jonas Salk[2] concerning the deterioration in antigenicity of some poliomyelitis virus vaccines which contained Merthiolate. When these preserved vaccines were subjected to increased temperatures in the incubator or to shipping conditions during the summer, the protective value of the vaccine was severely reduced in many instances. Although there was a significant variation of the effect of Merthiolate in the various vaccines, this preservative appeared to be deleterious to the vaccine. Either the Merthiolate had an unexpected direct action on poliomyelitis virus vaccine, or an unexpected set of conditions were being encountered in which Merthiolate was being degraded and was being rendered destructive to antigenicity.[3]

The antiseptic activity of Merthiolate (ethylmercurithiosalicylic acid, sodium salt) can be attributed to a partial ionization of the compound to give a low but effective level of ethyl mercuri ion ($C_2H_5Hg^+$), which blocks enzymatic processes by combining with sulfhydryl groups on the enzymes. This ionization is represented by the following equilibrium reaction:



The value of Merthiolate as a preservative over $C_2H_5Hg OH$ depends upon the fact that it provides a reservoir of $C_2H_5Hg^+$ at low concentration. The con-

From Lilly Research Laboratories, Indianapolis, Ind.
Received for publication July 6, 1955.
*Thimerosal, Eli Lilly Company.

centration of thiosalicylate ion obviously controls the level of free $C_2H_5Hg^+$ in the solution. A study of some of the factors which affect the stability of thiosalicylate and Merthiolate in solution has made it possible to solve the problem of the apparent incompatibility of Merthiolate and poliomyelitis vaccine.

MATERIALS AND METHODS

Merthiolate.—Merthiolate, 10 per cent aqueous solution, Mfg. Lot 606083, was kept in an amber-glass bottle under refrigeration as a stock solution.

Water.—Glass redistilled water was used for the physical and chemical studies in order to minimize the effects of trace metal ions.

Chemicals.—Analytical reagent grade chemicals were used for buffer salts and for reagents throughout, except as otherwise indicated. Triglycine and O,O′ dicarboxydiphenyl disulfide were prepared by the Lilly Laboratories.

Various Monovalent and Trivalent Poliomyelitis Virus Vaccines.—These were supplied by the Biological Production Division of this company. The vaccines were prepared from the Mahoney, MEFl, and Saukett strains of virus propagated on monkey kidney tissue culture, with subsequent inactivation of the virus by Formalin. The residual formaldehyde in some vaccines was neutralized according to the methods used by Salk[4]; such vaccines were referred to as ''neutralized.'' The neutralization procedure was omitted in the preparation of the vaccines referred to as non-neutralized. Some vaccines, neutralized and non-neutralized, had Merthiolate added in various concentrations. Various vaccines containing Merthiolate included additives such as gelatin and glycine, whereas others had the trisodium salt of ethylene diamine tetra-acetic acid (EDTA). The vaccines were tested before and after heating at 37° C. for various periods, generally up to 14 or 15 days.

Assay of Merthiolate in Solution.—Relative concentrations of Merthiolate were determined by comparing the ultraviolet absorbance of the solution at 250 and 280 mμ with the spectrum of a Merthiolate solution of known concentration. This control sample was used in each series of measurements. The ultraviolet spectra were determined with a recording spectrophotometer.*

Effects of Trace Metal Ions on Merthiolate.—An accelerated test to study the effects of different compounds on Merthiolate simply required holding the sample at elevated temperatures. The buffered solutions (pH 7.0 potassium phosphate or pH 8.7 sodium bicarbonate) containing 0.01 per cent Merthiolate and the compound expected to influence its stability were sealed in glass ampules. The ampules were heated in a boiling water bath for specified times. After chilling, the samples were diluted and subjected to ultraviolet analysis. Appropriate control samples were used simultaneously.

Assay of Merthiolate in Polio Vaccine.—Detection of subtle changes in the Merthiolate in vaccine required its extraction from the vaccine. Five milliliters of vaccine was acidified wth 0.2 ml. of 12 N HCl and extracted with 5 ml. of freshly distilled ether. After removal of the ether layer, the aqueous layer was again extracted with 2 ml. of ether. The combined ether solutions were shaken with 5 ml. of 1 per cent $NaHCO_3$. This bicarbonate solution was used for ultraviolet analysis.

Assays of Antigenicity.—In order to expedite immunologic tests of antigenicity of the various vaccines, considerable early use was made of the type 2 component and a one-dose mouse test. Information concerning the utility of this test was forwarded to us by Drs. Jonas Salk and Ulrich Krech,[5] prior to its formal publication by Krech.[6] A similar test for types 1 and 3 was developed by Powell and Culbertson.[7] Serum-virus neutralization tests against all three types of virus also were made in mice.[8] These sera were obtained from mice 1 week after 3 weekly doses of vaccine.

The challenge viruses which we have used in the one-dose mouse immunity tests of different poliomyelitis vaccine preparations (for example, special Mahoney, MEFl, and Leon strains) have already been described.[7] Mahoney, Lansing, and Leon strains were used in serum-virus neutralization tests conducted in mice.[8]

*Model 11 Cary.

Comparative Bacteriostatic and Fungistatic Action.—Some of the above lots of poliomyelitis vaccine preserved with Merthiolate in the presence of EDTA and glycine were utilized in comparative bacteriostatic and fungistatic tests. Test organisms included a laboratory strain of *Staphylococcus aureus* and four strains of fungi kept in these laboratories for use in chemotherapy tests. Such bacteriostatic and fungistatic tests were deemed necessary to prove the efficacy of Merthiolate as a preservative in the vaccine in the presence of these additives.

Effects of Thiosalicylate Ion on Fungicidal Activity of Merthiolate.—One hundred-ten-milliliter culture tubes containing sterile Sabouraud media were treated with 1 ml. of the test solutions before inoculation with a mixture of 13 yeasts. The tubes were left at room temperature and observed at intervals for signs of growth.

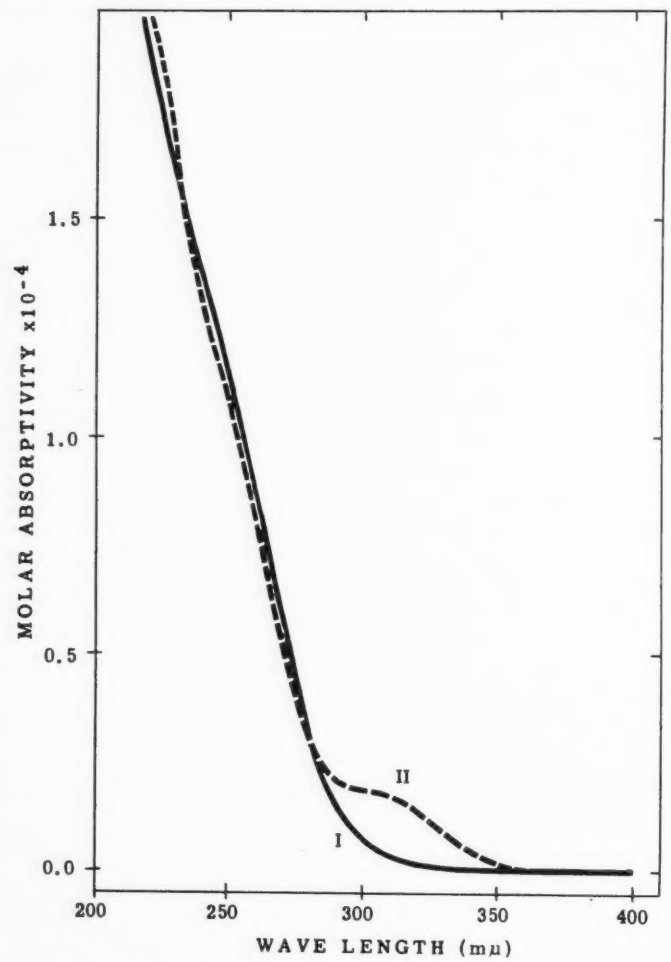


Fig. 1.—Ultraviolet spectra of 1.23×10^{-4}M aqueous Merthiolate solutions determined at pH 7 and 10 (Curve I) and at pH 2.0 (Curve II).

EXPERIMENTAL RESULTS

The Ultraviolet Absorption Curves of Merthiolate.—These are shown in alkaline, neutral, and acid solution in Fig. 1. Although the spectrum at neutral

pH does not have characteristic maxima and minima, the absorption is sufficiently intense to make ultraviolet absorption a feasible method of detecting Merthiolate in a solution if control solutions of known composition are used as standards.

The changes in the ultraviolet spectrum of Merthiolate resulting from heating the solutions in the presence of 1.0 p.p.m. Cu^{++} for various periods are illustrated in Fig. 2. This degradation of Merthiolate, as indicated by the reduced absorption near 250 mμ, was also proportional to the amount of Cu^{++} present in the solution. Sufficient amounts of metal ions were present in glass

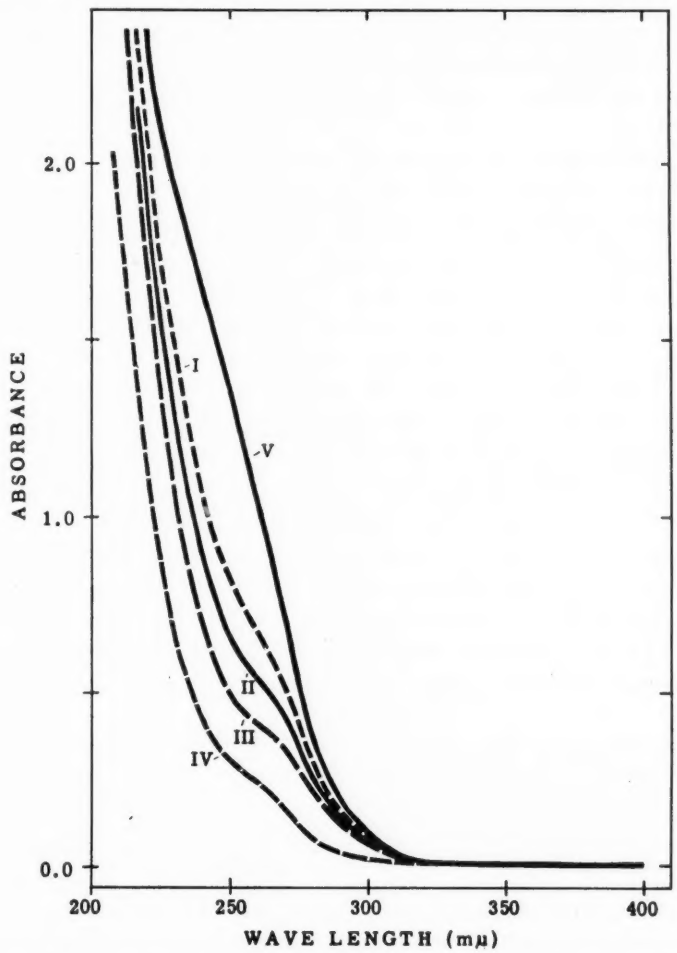


Fig. 2.—Effect of Cu^{++} on Merthiolate solutions. Changes in the ultraviolet spectrum of Merthiolate which demonstrate the chemical degradation of the compound. Merthiolate, 1.23×10^{-4}M, in 0.04M phosphate buffer, pH 7.0, and with 1 p.p.m. Cu^{++} was heated at 100° C. in sealed ampules for 5, 15, and 30 minutes (Curves I, II, and III, respectively). Curve V shows the spectrum before and after 30 minutes heating when 0.01 per cent EDTA was added to chelate the added Cu^{++}. Curve IV resulted from heating 1.23×10^{-4}M C_2H_5HgCl and 0.62×10^{-4}M O,O′ dicarboxydiphenyl disulfide for 30 minutes.

redistilled water and analytical reagent grade chemicals to catalyze this breakdown at 100° C. The ferric ion had less than 1 per cent of the catalytic effect of the cupric ion.

The ultraviolet absorption curve of the degraded Merthiolate was essentially reproduced by warming a solution of ethylmercuric chloride and O,O′ dicarboxydiphenyl disulfide at pH 7.0. (Curve IV of Fig. 2.) This indicates that Cu^{++} catalyzes the oxidation of thiosalicylate to the disulfide under these conditions.

After the addition of stoicheiometric quantities of EDTA to chelate the cupric ion, the Merthiolate remained stable in boiling water for prolonged periods. The ultraviolet spectra before and after 30 minutes of heating are reproduced as Curve V in Fig. 2. Although only one curve is shown, the spectrum of the heated sample coincided exactly with the unheated Merthiolate. Glycine afforded some protection against the effects of Cu^{++} but less than 5 per cent as effective as EDTA. Triglycine and gelatin were nearly inactive as stabilizing agents.

In order to determine the unchanged Merthiolate remaining in vaccines, the preservative was extracted from the acidified vaccine with freshly distilled ether. After shaking the ether solution with a bicarbonate solution, the aqueous phase was subjected to ultraviolet analysis. Typical ultraviolet spectra of samples extracted from vaccines subjected to different treatments are shown in Fig. 3. Freshly added Merthiolate was readily extracted from the vaccine (Curve I). When the preserved vaccine had been incubated for two weeks at 37° C., very little Merthiolate remained in an ether extractable form (Curve II). In fact, the ether soluble material was very nearly the same as that from the fresh vaccine without preservative (Curve III), indicating that the thiosalicylic acid, the ultraviolet absorbing structure of Merthiolate, had been changed to an ether insoluble compound. The addition of the EDTA at the same time as the Merthiolate, however, prevented the accelerated breakdown of the preservative. The ultraviolet absorbing material of this sample (Curve IV) after three months at 37° C. was quite similar to that of freshly added Merthiolate (Curve I), but at a reduced concentration. Curve V was from a sample of the same vaccine as used for Curve IV, but this vaccine was stored for three months at 5° C.

Stability of Type 2 Vaccine Component in One-Dose Mouse Tests.— Information gained from numerous exploratory tests led to the establishment of comparative immunity and stability tests of the type 2 component in trivalent poliomyelitis vaccine treated under controlled conditions as follows: vaccine produced, inactivated, and neutralized in the regular wave was divided into three samples. The first sample was used as the control without further treatment. Merthiolate was added to the second sample at a concentration of 1:10,000 (0.000247 molar). To the third portion were added Merthiolate at 1:10,000 and EDTA, trisodium salt, at a concentration of 0.00247 molar. After various periods of heating at 37° C. to accelerate deterioration, single 0.5 ml. doses of the various vaccines in fivefold dilutions were injected intraperitoneally into different groups of 10 mice. Three days later, these mice were given a challenge dose of 0.1 ml. of special MEF1 poliomyelitis virus intravenously. This dose, established by previous titrations, was intended to be about 10 PD_{50}, and was sufficient virus to kill or paralyze from 7 to 10 of a group of 10 normal

mice, included in every test. Furthermore, one-tenth of this challenge dose was sufficient usually to kill from 2 to 6 of another group of 10 normal mice. Precise determination of exact number of PD_{50} of virus used in each test is not necessary if the challenge dose is maintained in these limits. Seven days after the injection of the challenge virus, final readings were made and recorded in terms of mice appearing normal.

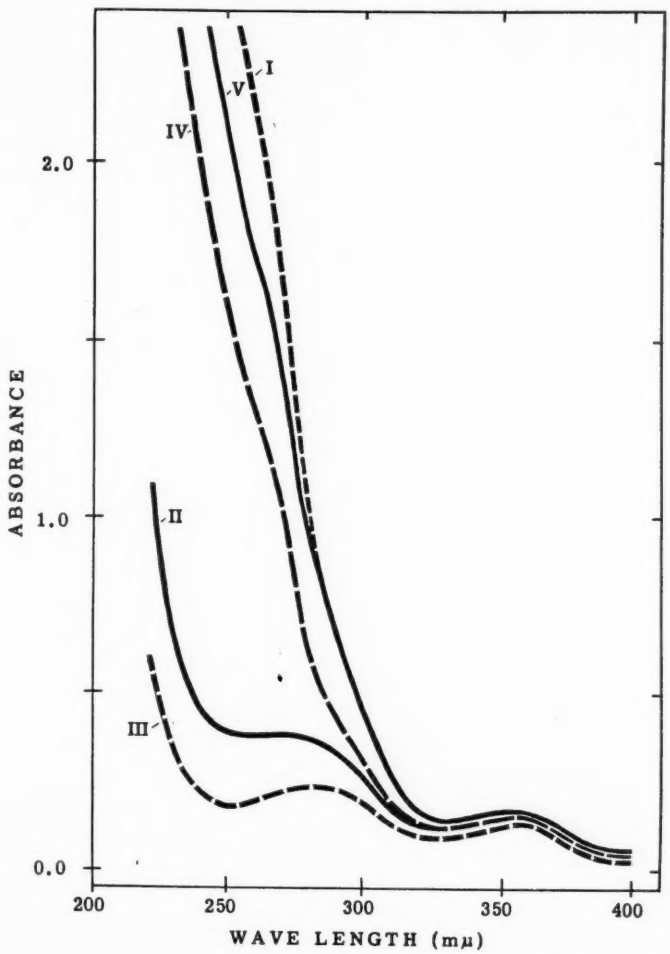


Fig. 3.—Stabilization of Merthiolate in vaccine. Ultraviolet spectra of bicarbonate solutions containing the ether-extractable material from acidified vaccines. When Merthiolate was used, the initial concentration was 0.01 per cent. Curve I, Freshly added Merthiolate. Curve II, Vaccine incubated 2 weeks at 37° C. with Merthiolate. Curve III, Unpreserved vaccine. Curve IV, Vaccine with Merthiolate and EDTA after 3 months at 37° C. Curve V, Vaccine with Merthiolate and EDTA after 3 months at 5° C.

Results of these first tests are shown in Table I. The unheated and undiluted vaccine protected 10 of 10 mice, and vaccine diluted 1:5 protected 5 of 10 mice against the challenge dose of virus which killed (or paralyzed) 9 of 10 controls. Samples of all three vaccines heated for 5 or 10 days, respectively, at 37° C. were included in this same test. It is noted from these results

that the vaccine sample preserved with Merthiolate 1:10,000 (vaccine 2684-B), has lost all of its type 2 potency after 10 days' heating. Vaccine without preservative (2684-A) and vaccine with Merthiolate and EDTA (2685-B) appear practically unaffected by heating. Subsequent tests of vaccine heated at 37° C. for eleven and fifteen days and certain repeat tests of vaccine heated fifteen days as indicated in Table I, gave the same general results; in other words, by using the type 2 component of trivalent vaccine as an index of stability, Merthiolate causes deterioration in potency, but the vaccine containing EDTA in addition to the Merthiolate immunizes as well as the unpreserved vaccine. Thus it became apparent in the early stages of this work that degradation products of Merthiolate, and not the Merthiolate molecule, were responsible for the loss of potency of the poliomyelitis vaccine.

TABLE I. STABILITY OF TYPE 2 COMPONENT OF TRIVALENT POLIOMYELITIS VACCINE ASSAYED BY ONE-DOSE MOUSE TEST

VACCINE	DILUTION	NORMAL MICE/MICE USED, SEVEN DAYS AFTER CHALLENGE							
		0 DAYS	5 DAYS	10 DAYS	11 DAYS	15 DAYS	15 DAYS (REPEAT)	15 DAYS (REPEAT)	15 DAYS (REPEAT)
2684-A	Str.	10/10	—	7/10	—	9/10	—	—	—
No Merthiolate	1:5	5/10	—	5/10	—	7/10	—	—	6/9
	1:25	—	—	—	—	—	—	4/10	5/10
	1:125	—	—	—	—	—	—	1/10	—
2684-B	Str.	—	—	1/10	—	1/10	—	—	—
Merthiolate	1:5	—	—	1/10	—	1/10	—	—	—
1:10,000	1:25	—	—	—	—	—	—	—	—
	1:125	—	—	—	—	—	—	—	—
2685-B	Str.	—	7/10	—	8/10	—	5/9	—	—
Merthiolate	1:5	—	8/10	—	8/10	—	7/10	—	6/10
1:10,000	1:25	—	—	—	—	—	—	6/10	7/10
EDTA 0.00247 M	1:125	—	—	—	—	—	—	4/10	—
Virus virulence controls	1 challenge dose	1/10	1/10	1/10	0/10	0/10	2/10	3/10	0/10
	0.1 challenge dose	7/10	7/10	7/10	2/10	2/10	8/10	7/10	5/10

Different groups of 10 mice each received a single intraperitoneal dose of 0.5 ml. of the indicated vaccine dilutions and 3 days later were challenged intravenously with 0.1 ml. of 10^{-1} virus. The vertical columns indicate results with vaccines heated at 37° C. for various numbers of days.

Many different production lots of vaccine were tested for comparative type 2 response in mice using the same triad of samples (vaccine alone, vaccine with Merthiolate, and vaccine with Merthiolate and EDTA) and different periods of heating. These tests proved to be feasible and rapid in assessing various production methods in large scale preparation of vaccine.

Stability Tests of All 3 Components of Neutralized Trivalent Vaccine in N.I.H. Three-Dose Mouse Tests.—The results just described from the one-dose mouse tests with the type 2 component in trivalent poliomyelitis vaccine have been duplicated in the N.I.H. three-dose vaccine test in mice in which immunity against all 3 types of virus is determined. In these tests, groups of 20 mice are injected with 3 weekly intraperitoneal doses of 0.5 ml. of vaccine in fivefold dilutions, and one week after the third dose the mice are bled and the pooled

sera from the groups are subjected to serum-virus neutralization tests in additional mice. Serum-virus dilutions involving types 1 and 3 are injected intraspinally into groups of 10 mice for each serum dilution; but for type 2 virus, the serum-virus dilutions are injected intracerebrally into similar groups of mice. Test doses of virus per mouse comprise about 10 PD_{50} for types 1 and 3 and about 40 PD_{50} for type 2. After final reading of the tests, the serum dilution saving 50 per cent of the mice is computed. Table II gives the results of this test applied to the same three heated vaccines tested previously by the one-dose test. In Table II it is noted that vaccine 2684-A after 15 days at 37° C. and without preservative immunizes mice sufficiently to show definite serum antibody titers to all three types of virus. Contrastingly, vaccine 2684-B heated with Merthiolate 1:10,000 does not immunize appreciably. However, when 0.00247 molar EDTA is added to Merthiolate 1:10,000 vaccine 2685-B, normal immunization of the mice against all three types of virus is attained with the heated vaccine. Thus, it has again been demonstrated that the addition of 10 moles of EDTA per mole of Merthiolate prevents the decrease in protective value of the poliomyelitis vaccine resulting from the addition of Merthiolate alone.

TABLE II. STABILITY OF TRIVALENT POLIOMYELITIS VACCINE ASSAYED BY MOUSE IMMUNIZATION AND SERUM NEUTRALIZATION TESTS*

VACCINE (HEATED 15 DAYS AT 37° C.)	THREE WEEKLY DOSES OF 0.5 ML. VACCINE DILUTED	SERUM-VIRUS NEUTRALIZATION TEST OF SERUM FROM MICE IMMUNIZED AS SHOWN IN COLUMN 2 (COMPUTED SERUM DILUTION PROTECTING 50% OF MICE)		
		TYPE 1	TYPE 2	TYPE 3
2684-A	Undiluted	16.8	6.3	8.6
No Merthiolate	1:5	7.5	5.6	5.7
2684-B	Undiluted	<4	<4	<4
Merthiolate 1:10,000	1:5	<4	<4	<4
2685-B	Undiluted	19.4	7.3	8.5
Merthiolate 1:10,000 + EDTA 0.00247 M	1:5	4.5	4.3	6.0

*Tests made as described by N. I. H. Minimum Requirements.

Stability of Trivalent Vaccine Including Different Concentrations of Merthiolate Without EDTA.—In similar experiments, it was observed that the type 1 component in neutralized trivalent poliomyelitis vaccine falls in potency somewhat faster in heat stability tests of vaccine preserved with Merthiolate alone than the types 2 and 3 components. At a concentration of 1:10,000, Merthiolate causes severe damage to the type 1 component and partial damage to the type 2 component after 5 days of heating at 37° C. Only slight loss in antigenicity of type 1 occurs during the same period if 1:20,000 Merthiolate is used, whereas 1:40,000, 1:50,000, and 1:100,000 are nearly without effect. However, if the period of heating is increased to 15 days, 1:100,000 Merthiolate causes appreciable loss of potency of the type 1 component.

Effects of Different Concentrations of EDTA.—In earlier experiments, 10 moles of EDTA were used for each mole of Merthiolate. Since the optimum

concentration of the stabilizing agent may be related to the amount of heavy metal ions in the solution and not to the amount or concentration of Merthiolate, experiments were conducted to determine the margin of safety which a concentration of EDTA of 0.00247 M would have in stability tests of 2 weeks at 37° C. under the conditions surrounding the preparation of vaccine in these laboratories. For this purpose, three fivefold decreasing concentrations of EDTA (0.00049 M, 0.000098 M, and 0.000019 M, respectively) were used in three lots of vaccine which were heated for 7 and 14 days at 37° C. Potency of the type 2 component in these three vaccines was determined by the one-dose vaccine test in mice with direct challenge 3 days later. The results are shown in Table III. If 5 to 10 mice of groups of 10 live, the vaccine is considered reasonably immunogenic. It appears, especially from the results in the 14 day column, that EDTA 0.000019 M, or 0.08 mole per 1 mole Merthiolate, is not sufficient, whereas 0.000098 M, or 0.4 mole EDTA per 1 mole Merthiolate is the minimal concentration of stabilizing agent necessary. This amount is one-twenty-fifth the amount used in the test described above; hence, there is a twenty-fivefold safety factor in terms of stability of the type 2 component in the vaccine. Since the type 1 component is somewhat more labile than the type 2, this safety factor would be less, but still adequate, for type 1. EDTA, 0.00247 M, alone added to neutralized vaccine has not been found to affect the potency of the vaccine when heated at 37° C. for 15 days.

TABLE III. EFFECT OF CONCENTRATION OF EDTA ON HEAT STABILITY OF TYPE 2 COMPONENT OF POLIOMYELITIS VACCINE PRESERVED WITH MERTHIOLATE 1:10,000

VACCINE	EDTA	MOLES EDTA PER MOLE MERTHIOLATE	DIL. OF VACCINE	NORMAL MICE/MICE USED 7 DAYS AFTER CHALLENGE		
				0 DAYS	7 DAYS	14 DAYS
2751	None (also no		Str.	10/10	—	—
(Parent lot)*	Merthiolate)	—	1:5	5/10	—	—
			1:25	—	—	—
2759-A			Str.	—	10/10	—
Merthiolate 1:10,000	0.00049 M	2	1:5	—	8/9	5/10
			1:25	—	9/9	4/10
2759-B			Str.	—	10/10	—
Merthiolate 1:10,000	0.000098 M	0.4	1:5	—	10/10	7/10
			1:25	—	10/10	3/10
2759-C			Str.	—	9/10	—
Merthiolate 1:10,000	0.000019 M	0.08	1:5	—	9/10	2/10
			1:25	—	7/10	2/10
Virus virulence	1 challenge dose			1/10	2/10	1/10
controls	0.1 challenge dose			7/10	7/10	5/10

One-dose mouse test used for assay. Columns at right show days of heating of vaccine at 37° C. prior to immunization.

*Same vaccine as 2684-A in Table I where heat stability is shown.

Gelatin at 1:1,000 and 0.3 M glycine were also tried as a protective agent for the vaccine. Although some stability was afforded by these compounds, 14 days of incubation at 37° C. caused an appreciable loss of activity in these vaccines.

Lack of Stability of Nonneutralized Vaccines Containing Merthiolate and EDTA.—The excess formaldehyde in all the vaccines referred to above had been neutralized with sodium bisulfite. Vaccine preparations similar to those referred to above, but comprising "nonneutralized" samples, have been used in parallel stability tests similar to the heating experiment indicated with the neutralized vaccines. In contrast to the neutralized vaccine, the nonneutralized vaccines with the Merthiolate preservative and EDTA suffer a loss in potency in heat stability tests at 37° C. It is believed these results are so consistently negative, either at 7 or 14 days' heating, that detailed results need not be tabulated here. It is impractical to use nonneutralized nonpreserved poliomyelitis vaccine since the residual formaldehyde per se causes loss of antigenicity (first of the type 1 component, then of the other types) and this is readily demonstrable in stability tests conducted at 37° C. Furthermore, such vaccine does not show inhibitory action against mold growth.

On the Lack of Interference of EDTA on Antiseptic Action of Merthiolate.—Tests were conducted to determine if addition of EDTA along with Merthiolate to poliomyelitis vaccine, as has been described for the stabilization of the vaccine solution, would affect the otherwise strong antiseptic activity of Merthiolate.[2] Serial dilutions of various samples of poliomyelitis vaccine containing Merthiolate 1:10,000 (0.000247 M) and varying EDTA concentrations were made in Pen assay broth, and 0.1 ml. of a 24-hour broth culture of *Staphylococcus aureus* was planted into 5 ml. amounts of these dilutions. Readings of turbidity were made after 24 hours' incubation at 37° C. Concentrations of EDTA from 1 to 100 moles per mole of Merthiolate did not affect the antistaphylococcal bacteriostatic action of Merthiolate. A second reading at 85 hours showed the same results as the 24-hour reading except in one tube which showed some growth in the presence of 1:1,600,000 Merthiolate.

Additional tests were done against certain fungi, with the same general result as follows: Dilutions of vaccines, both neutralized and nonneutralized, containing EDTA (0.00247 M), glycine (0.3 M), and EDTA and preserved with Merthiolate 1:10,000 were made up in medium 199; similar dilutions of Merthiolate 1:10,000, alone, were made up in medium 199. These serial doubling dilutions were planted with a heavy dose of spores of *Penicillium frequentis, Fusarium moniliforme, Mucor genevensis,* and *Aspergillus niger.* The tubes were left standing at room temperature for 10 days before readings were made. Spore controls in medium 199 grew out in 1 or 2 days. The fungistatic titers of the preparations containing Merthiolate and EDTA were the same as those without EDTA. These data agreed with our results on the antifungal action of Merthiolate published in 1934.[9] It appears there is no suppression of the fungistatic or bacteriostatic activity of Merthiolate by EDTA.

Effect of Thiosalicylate Ion on Merthiolate.—Twenty-five parts per million of Merthiolate completely inhibited growth of a mixture of 13 yeasts in Sabouraud media for 14 days. The addition of 8×10^{-3} M thiosalicylate ion sufficiently reduced the fungistatic activity of Merthiolate at 100 parts per million for growth to appear after seven days. Although the rate of growth

of yeasts in the tubes containing the sodium thiosalicylate and Merthiolate was inversely proportional to the amount of Merthiolate used, the addition of thiosalicylate ion prevented complete inhibition of growth by concentrations of Merthiolate otherwise fungistatic. This lends support to the mechanism expressed by Equation I, since a high concentration of thiosalicylate ion would reduce the concentration of $C_2H_5Hg^+$.

DISCUSSION

Very early in the use of Merthiolate as an antiseptic for human application, it became apparent that the shelf life of this drug could be deleteriously affected if trace amounts of copper (from one-half part to two parts or more per million) by chance might be included in the solvent. Copper, in this instance, was found to catalyze the oxidation of Merthiolate to ethyl mercuri hydroxide and related degradation products (all more caustic than the Merthiolate molecule) perhaps a hundredfold, or more, faster than was the case in solutions nearly entirely free of copper. The marketed antiseptic solution has, in the past, been stabilized with ethylene diamine and ethanolamine. These two compounds, however, were ineffective in protecting the poliomyelitis vaccine from the deleterious effects of the preservative.

Biologic materials usually contain sufficient amounts of proteinaceous materials (broth, peptone, serum proteins, and amino acids) to adsorb trace amounts of heavy metals, especially copper, which are usually present at low levels. In these biologics, Merthiolate, added as a preservative, is not usually subjected to degradation. Also, the extra protein probably offers some protection against the breakdown products of Merthiolate. Unusual amounts of heavy metals or low content of protein (or both these conditions) in a vaccine would warrant attention regarding vaccine stability toward the preservative. Poliomyelitis trivalent tissue culture vaccine contains very small amounts of nitrogenous substances, (about 0.35 mg. total nitrogen per milliliter) of which 0.25 mg. is derived from medium 199. This lack of protection afforded by an excess of protein offers a possible explanation to the sensitivity of the poliomyelitis vaccine degradation by nonstabilized Merthiolate.

Although the procedures for poliomyelitis vaccine production are rigidly controlled, the amount of heavy metals in the finished vaccine could vary several fold without being present in concentrations detectable by normal analytic methods. To insure against the degradation of the Merthiolate in poliomyelitis vaccines, our vaccine has a 25-fold excess of EDTA added over the minimum concentration, which gives complete stabilization of this Salk poliomyelitis vaccine.

The failure of EDTA to prevent the loss of potency of the vaccine which has excess formaldehyde and Merthiolate may be due to a sensitization of the virus particle to formaldehyde by the very low concentration of $C_2H_5Hg^+$ formed in the dissociation of Merthiolate. Since residual formaldehyde alone weakens the potency of the incubated vaccine, it was deemed necessary to neutralize the excess formaldehyde in all the poliomyelitis vaccine.

SUMMARY

1. Solutions of Merthiolate are stable to prolonged periods of heating when traces of cupric ion are removed.

2. Trivalent poliomyelitis virus vaccine produced in monkey kidney tissue culture may be satisfactorily preserved with Merthiolate if the trisodium salt of ethylene diamine tetra-acetic acid is added.

3. The protective antigenicity of the vaccine against the three types of virus is maintained in accelerated aging tests at 37° C. for two weeks.

4. The antibacterial and antifungal actions of this preservative are not diminished by the addition of EDTA.

REFERENCES

1. Powell, H. M., and Jamieson, W. A.: Merthiolate (Thimerosal, Lilly) as a Germicide, Am. J. Hyg. **13**: 296-310, 1931.
2. Personal communication in October, 1954, from Dr. Jonas Salk.
3. Stabilized organo-mercuri-sulfur compounds, Moris S. Kharasch, U. S. Patent 2,012,820.
4. Salk, J.: Studies in Human Subjects on Active Immunization Against Poliomyelitis. J. A. M. A. **151**: 1081, 1953.
5. Personal communication from Drs. Jonas Salk and Ulrich Krech.
6. Krech, U.: Development of Immunity in Mice After Vaccination With Inactivated Poliomyelitis Virus. J. Immunol. **74**: 117-119, 1955.
7. Powell, H. M., and Culbertson, C. G.: Mouse Immunity Tests of Trivalent Poliomyelitis Vaccine, Proc. Soc. Exper. Biol. & Med. **88**: 563-564, 1955.
8. See Minimum Requirements; Poliomyelitis Vaccine. Amendment No. 1 of May 20, 1954, issued by the National Institutes of Health.
9. Powell, H. M., and Jamieson, W. A.: On Merthiolate and Fungi Associated With Ringworm, Proc. Ind. Acad. Sc. **43**: 56-70, 1934.

PREPARATION OF GLYCYRRHIZINIC ACID, THE ELECTROLYTE-ACTIVE PRINCIPLE OF LICORICE: ITS EFFECTS UPON METABOLISM AND UPON PITUITARY-ADRENAL FUNCTION IN MAN

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IN 1946 Revers[1] observed that many patients suffering from peptic ulcer were benefited by ingesting a crude, watery extract of licorice. He noted, too, that about 20 per cent of the patients so treated gained weight and developed edema of the ankles. Molhuysen and associates[2] extended this work and demonstrated that licorice extract when given orally to normal persons causes retention of sodium and chloride as well as excretion of potassium. Groen and co-workers[3] and Card and associates[4] reported beneficial effects in patients with Addison's disease fed licorice extract, but upon this point there was lack of agreement.[2, 5]

Recently Borst and associates[5] have put forth the hypothesis that licorice is active only in the presence of a small amount of glucocorticoid. They observed in patients with Addison's disease that licorice was without effect but that doses of cortisone (2.5 mg.) would restore partially the electrolyte activity of the licorice. When 10 mg. of cortisone was given with licorice, the electrolyte effect of the latter was wholly restored.

The fact that the DOC-like effects of different commercial preparations of licorice are dissimilar could explain some of the apparently contradictory results. Attempts to fractionate and identify the active principle were not successful.[3-6] As far as we are aware, no chemically pure compound or compounds isolated from licorice have been studied except that reported recently by Pelser and associates.[7] They administered 6 Gm. of pure glycyrrhizinic acid (4 Gm. on the first day and 2 Gm. of the pure substance plus 2 Gm. of a crude preparation on the second day) to a patient with Addison's disease and observed significant retention of sodium and chloride.

The purpose of the present communication is to outline a simple method for the isolation of pure ammonium glycyrrhizinate in large yield from licorice extract and to report upon the metabolic and physiologic effects of this compound in man. The subjects of this investigation consisted of seven normal

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persons, two patients with congenital adrenal hyperplasia, and one patient with Cushing's syndrome due to bilateral adrenal hyperplasia (demonstrated at subsequent operation).

METHODS

1. *Preparation of Ammonium Glycyrrhizinate.*—The procedure of isolation of ammonium glycyrrhizinate from licorice is a modification of the method of Bergmann.[8] To 1.5 L. of hot glacial acetic acid (60° to 70° C.), 400 Gm. of ammoniated glycyrrhizin* is added slowly, as the mixture is vigorously stirred. After it has been stirred for about one hour at 60° to 70° C., the solution is allowed to stand at room temperature for a day or two. The mixture is filtered through a large Buchner funnel and washed with cold glacial acetic acid and finally with anhydrous ether.

The dark amorphous precipitate is placed in an evaporating dish and allowed to dry in a vacuum desiccator. After the material is somewhat dried, it is dissolved in 600 ml. of hot 80 per cent ethyl alcohol and heated in a steam bath for fifteen to twenty minutes. The solution is then placed in the refrigerator overnight for crystallization.

The semicrystalline solid is filtered through a Buchner funnel and recrystallized four or five times in 80 per cent ethyl alcohol. The brown color is removed by boiling the solution with norit several times during the purification process. The yield is 20 to 22 Gm. From the alcoholic filtrate, an additional 10 to 15 Gm. of the pure substance is obtained. The product is a snow-white crystalline substance and has the typical licorice taste. The formula and analysis† of the compound are given in Fig. 1.

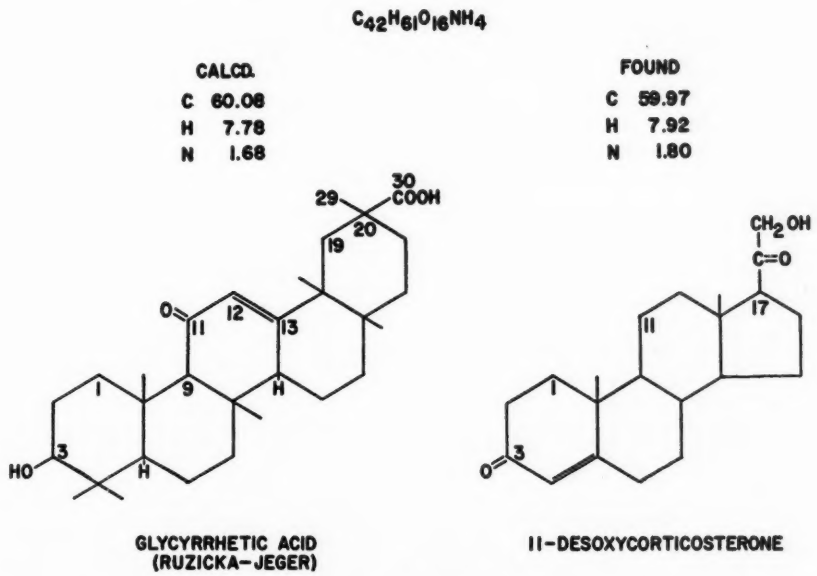

Fig. 1.—Analysis of ammonium glycyrrhizinate.

2. *Experimental Subjects and Procedure.*—Three men and four women constituted the group of normal subjects. They, as well as the patients (two girls with congenital adrenal hyperplasia and one woman with Cushing's syndrome), ingested a constant diet of average composition. In accordance with the rule of this laboratory, the subjects ingested the diet for at least three days before control data were obtained.

After a suitable control period had been obtained, ammonium glycyrrhizinate was administrated orally four times daily (8 A.M., 12 M., 6 P.M., and 11 P.M.). The amounts

*The Polychemical Company, New York, N. Y.
†Schwarzkoph Microanalytical Laboratory, Long Island, N. Y.

administered and the duration of administration of this active compound are indicated in the various figures. Fasting blood sugar, hematocrit, total circulating eosinophil cells, blood pressure, and body weight were determined daily. Glucose tolerance tests were performed before, during, and following administration of the compound. Serum sodium, potassium, and chloride were determined at frequent intervals. Total daily urinary excretion of sodium, potassium, chloride, glucose, uric acid, nitrogen, and 17-ketosteroids was determined. A series of samples of thermal sweat were collected from two of the normal subjects. These were analyzed for their electrolyte composition.

The chemical methods used in the various determinations have been described previously.[9, 10] Sodium and potassium were determined in a Beckman flame photometer.

RESULTS

1. *Effects of Ammonium Glycyrrhizinate Upon Organic Metabolism.*—In none of the ten subjects studied was any significant effect observed upon protein or carbohydrate metabolism; nor was any effect demonstrated upon the renal excretion of uric acid and creatinine or upon the level of circulating eosinophils.

2. *Effect of Ammonium Glycyrrhizinate Upon Electrolyte Metabolism.*—Here very significant effects were demonstrable on all normal subjects although considerable variation in the intensity of effects was observed from subject to subject.

The data on four of the normal subjects (typical of all) are presented in Figs. 2 to 5. The most intense effect is upon retention of sodium and chloride. In contrast with the findings of the Dutch workers,[2] we observed only a small excretion of extra potassium. Upon withdrawal of ammonium glycyrrhizinate, there occurred slight retention of potassium and great loss of sodium and chloride. Body weight did not return to original levels for five to seven days following cessation of administration of the compound.

The patient with Cushing's syndrome retained sodium and chloride and lost potassium when given the compound, as did the normal subjects. It is of considerable interest that no effect of ammonium glycyrrhizinate upon electrolyte metabolism was demonstrable in either of the cases of congenital adrenal hyperplasia. Yet in all three situations (normal subjects, Cushing's syndrome, and congenital adrenal hyperplasia), the compound appears to depress endogenous ACTH (see following).

3. *Effect of Ammonium Glycyrrhizinate Upon Excretion of 17-Ketosteroids.*—In these experiments it was demonstrated repeatedly that 17-ketosteroid excretion could be depressed by administration of the compound. This phenomenon was observed most clearly in normal subjects with high levels of 17-ketosteroid excretion and in the patients with congenital adrenal hyperplasia.

The effect of ammonium glycyrrhizinate on the renal excretion of 17-ketosteroids on three normal subjects, the two patients with adrenogenital syndrome, and the case of Cushing's syndrome is presented in Figs. 6 to 9.

Fig. 6 demonstrates that administration of the compound produces a significant decrease of urinary 17-ketosteroids in normal persons. Clearly, this is not the degree of depression which can be produced by administration of some adrenocortical compounds. Of the remaining four normal subjects

(Figs. 2 to 5), three showed a decrease of 17-ketosteroid excretion while one showed no change (Fig. 5). In the latter case control values were below 5 mg. per day.

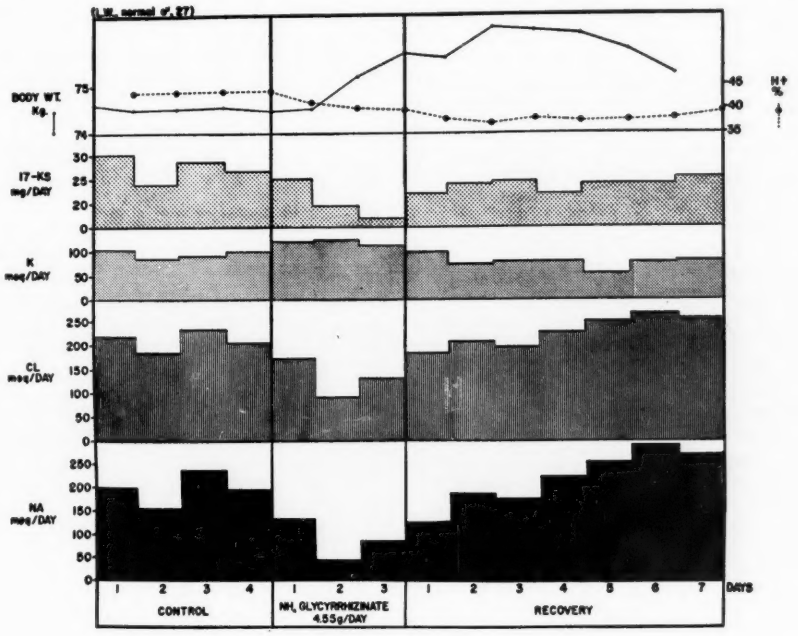

Fig. 2.

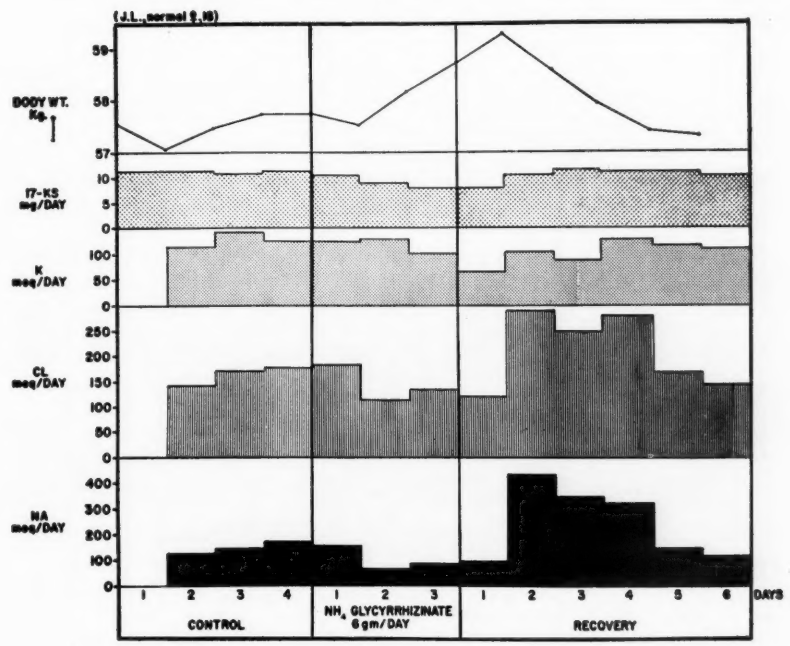

Fig. 3.

Figs. 2 and 3.—Effect of ammonium glycyrrhizinate on the renal excretion of sodium, chloride, potassium, and 17-ketosteroids.

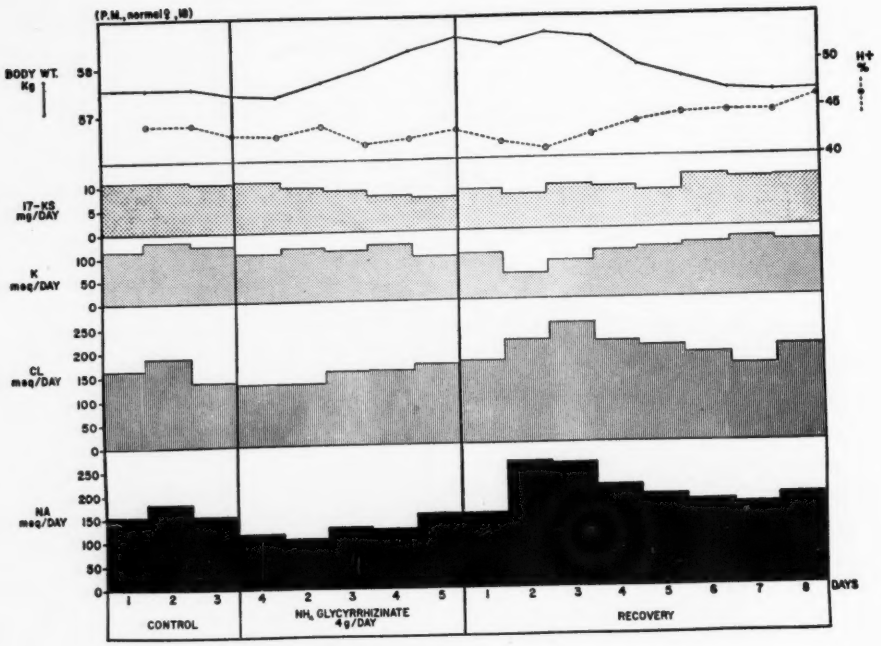

Fig. 4.

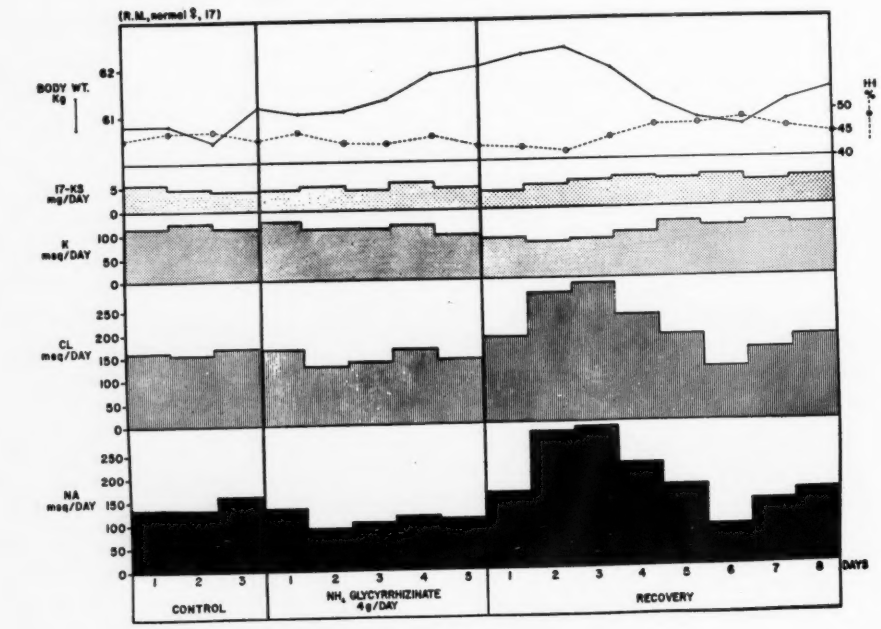

Fig. 5.

Figs. 4 and 5.—Effect of ammonium glycyrrhizinate on the renal excretion of sodium, chloride, potassium, and 17-ketosteroids.

In the case of congenital adrenal hyperplasia with very high levels of 17-ketosteroids (Fig. 7), the depressing effect of the compound is much more clearly demonstrated. It is equally clear that 120 mg. of cortisone (30 mg. orally every six hours) is considerably more effective in this respect.

The second case of congenital adrenal hyperplasia (Fig. 8) shows the effect on 17-ketosteroid excretion of increasing doses of ammonium glycyrrhizinate. Again the reduction of urinary 17-ketosteroids is evident.

In the case of Cushing's syndrome with normal base-line values for 17-ketosteroids (Fig. 9), administration of the compound for seven days (left side of the figure) and again for thirty-one days (right side of the figure) reduced 17-ketosteroid excretion only mildly, but definitely.

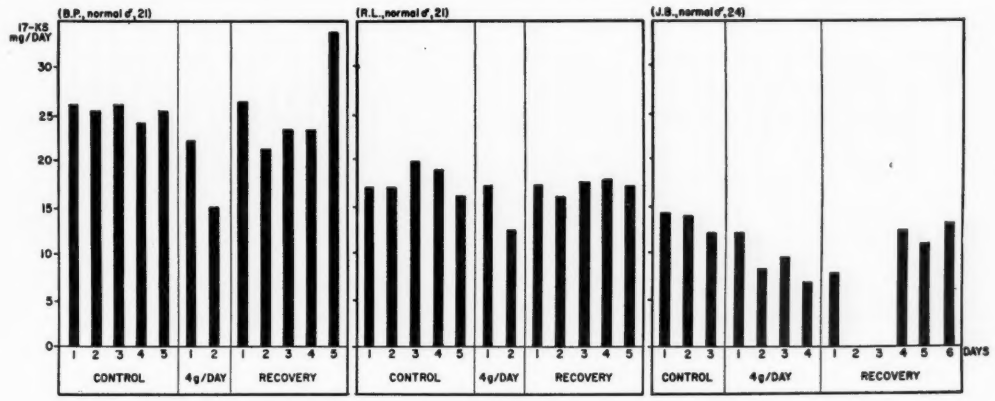

Fig. 6.

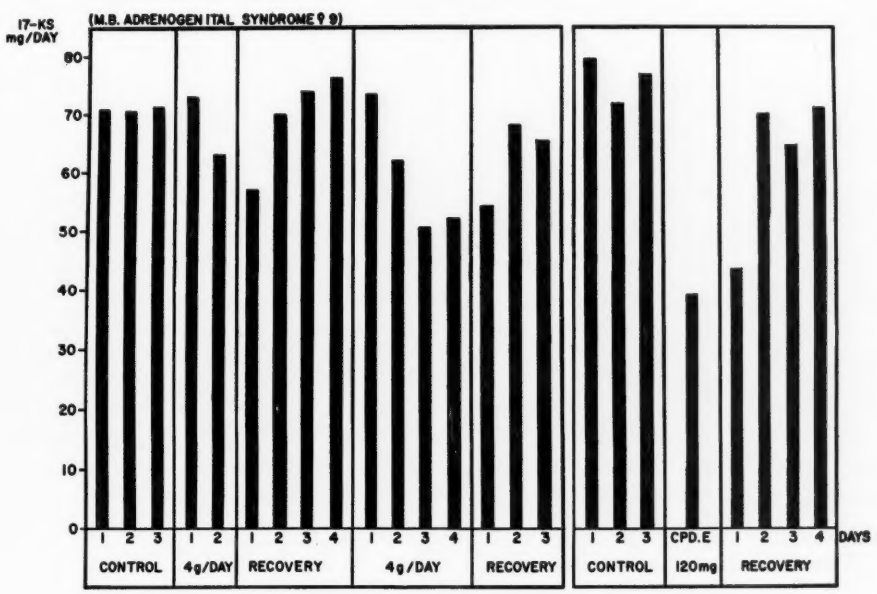

Fig. 7.

Figs. 6 and 7.—Effect of ammonium glycyrrhizinate on the renal excretion of 17-ketosteroids.

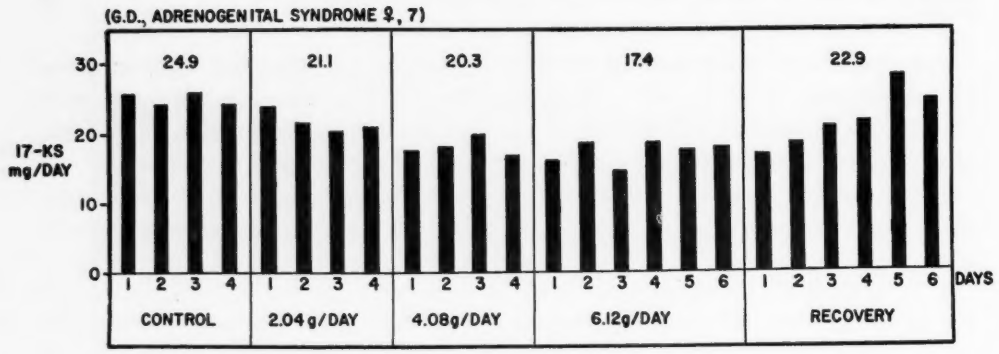

Fig. 8.

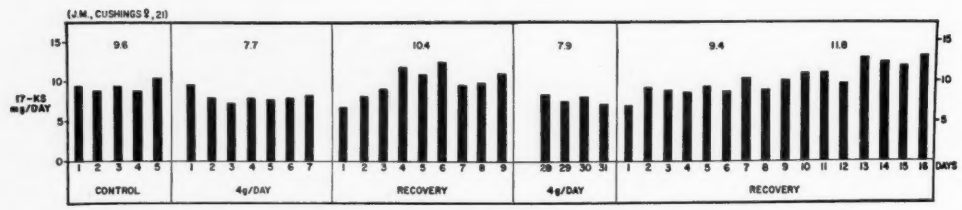

Fig. 9.

Figs. 8 and 9.—Effect of ammonium glycyrrhizinate on the renal excretion of 17-ketosteroids.

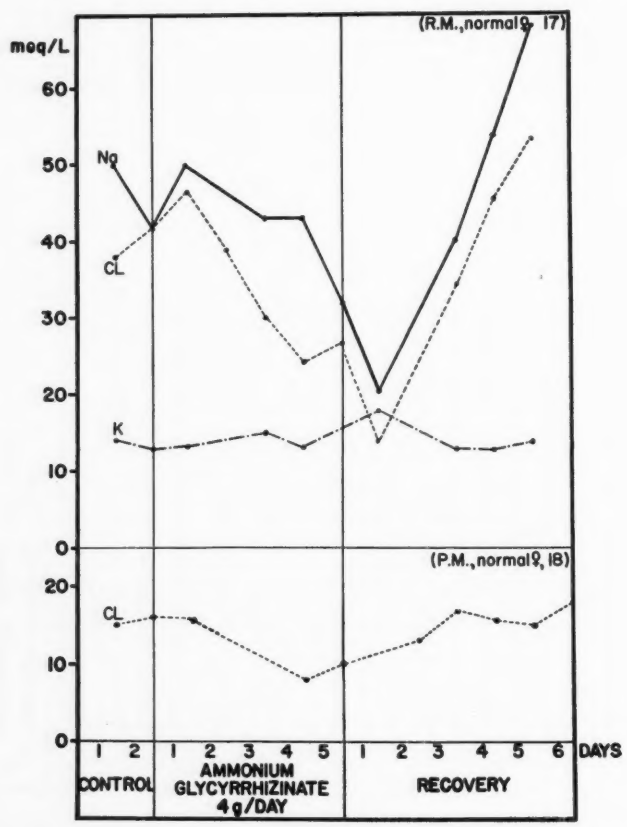

Fig. 10.—Effect of ammonium glycyrrhizinate on sweat electrolytes.

4. *Effect of Ammonium Glycyrrhizinate Upon Electrolyte Composition of Thermal Sweat.*—Since those adrenal steroids which exhibit intense sodium-retaining activity influence significantly the electrolyte composition of thermal sweat,[10-14] the effect of ammonium glycyrrhizinate in this respect was explored. Fig. 10 demonstrates that the results are those which would be expected of a potent sodium-retaining adrenal cortical steroid. On Subject P. M., only the chloride concentration of sweat was determined. The decrease in concentration of sweat chloride was much less than in Subject R. M., but the base-line values were already at the lower limit of normal.

5. *Effect of Ammonium Glycyrrhizinate Upon Urinary Excretion of Melanocyte-Stimulating Hormone (MSH).*[15, 16*]—A patient (alopecia areata) with abnormally high levels of urinary MSH was given ammonium glycyrrhizinate, 4 Gm. per day orally for a period of ten days. By the fourth day urinary MSH had fallen to normal levels. Within one day following cessation of administration of the compound, urinary MSH was back to its original high value. With respect to this activity (inhibition of pituitary release of MSH), 4 Gm. per day of this compound was found to be roughly equivalent to 5 mg. per day of 9-alpha fluorohydrocortisone, 20 mg. per day of hydrocortisone, and 37.5 mg. per day of cortisone. All these tests were carried out in the same patient.

CONCLUSIONS

Ammonium glycyrrhizinate, the sodium-retaining principle of licorice, has the following metabolic and physiologic effects when administered orally to man:

1. It induces great retention of sodium, chloride, and water.
2. It produces a mild increase of urinary potassium.
3. It inhibits mildly endogenous production of ACTH as indicated by consistent decreases in the excretion of 17-ketosteroids.
4. It decreases the concentrations of sodium and of chloride in thermal sweat.
5. It is capable of inhibiting pituitary release of MSH (melanocyte-stimulating hormone), the effect of 4 Gm. per day orally being roughly equivalent to 37.5 mg. of cortisone, 20 mg. of hydrocortisone, and 5 mg. of 9-alpha fluorohydrocortisone given orally per day, respectively.
6. It provokes no demonstrable effects upon organic metabolism (protein, carbohydrate, uric acid, creatinine, or circulating eosinophils).

The various demonstrable effects are highly suggestive that ammonium glycyrrhizinate is acting within the body in a way very similar to an adrenal cortical steroid; that its peripheral effects are exclusively upon electrolyte and water metabolism but that it is, nevertheless, capable of depressing endogenous production of both ACTH and MSH. Whether these activities are inherent in the compound as administered or result from metabolic transformations of the compound within the body must await more detailed studies. The

*We are greatly indebted to Dr. A. B. Lerner, University of Oregon Medical School, for the data described here.[17]

possibility of an hepatic metabolic transformation seems likely since we have failed to elicit any effects when as much as 90 mg. per day was administered intramuscularly. This result, however, may prove to be simply a matter of dosage.

REFERENCES

1. Revers, F. E.: Heeft Succus Liquiritiae een genezende werking op de Maagzweer? Nederl. Tijdschr. Geneesk. **90:** 135-137, 1946.
2. Molhuysen, J. A., Gerbrandy, J., de Vries, L. A., de Jong, J. C., Lenstra, K. B., Turner, K. P., and Borst, J. G. G.: A Liquorice Extract With Desoxycortone-like Action, Lancet **2:** 381-386, 1950.
3. Groen, J., Pelser, H., Willebrands, A. F., and Kamminga, C. E.: Extract of Licorice for the Treatment of Addison's Disease, New England J. Med. **244:** 471-475, 1951.
4. Card, W. I., Strong, J. A., Tompsett, M. W., Taylor, N. R. W., and Wilson, J. M. G.: Effects of Liquorice and Its Derivatives on Salt and Water Metabolism, Lancet **1:** 663-668, 1953.
5. Borst, J. G. G., Ten Holt, S. P., de Vries, L. A., and Molhuysen, J. A.: Synergistic Action of Liquorice and Cortisone in Addison's and Simmond's Disease, Lancet **1:** 657-663, 1953.
6. Groen, J., Pelser, H. E., Frenkel, M., Kamminga, C. E., and Willebrands, A. F.: Effect of Glycyrrhizinic Acid on the Electrolyte Metabolism in Addison's Disease, J. Clin. Invest. **31:** 87-91, 1952.
7. Pelser, H. E., Willebrands, A. F., Frenkel, M., van der Heide, R. M., and Groen, J.: Comparative Study of the Use of Glycyrrhizinic and Glycyrrhetinic Acids in Addison's Disease, Metabolism **2:** 322-334, 1953.
8. Bergmann, F.: Über das Enzymatische Verhalten von Gluroniden und über Glycirrhizinsaure, Biochem. Ztschr. **267:** 296-308, 1933.
9. Fajans, S. S., Louis, L. H., and Conn, J. W.: Metabolic Effects of Compound S (11-desoxy-17-hydroxy-corticosterone) in Man, J. Lab. & Clin. Med. **38:** 911-915, 1951.
10. Conn, J. W.: Electrolyte Composition of Sweat: Clinical Implications as an Index of Adrenal Cortical Function, Arch. Int. Med. **83:** 416-428, 1949.
11. Conn, J. W., Johnston, M. W., and Louis, L. H.: Acclimatization to Humid Heat: A Function of Adrenal Cortical Activity, J. Clin. Invest. **25:** 912-913, 1946.
12. Conn, J. W., and Louis, L. H.: Production of Endogenous "Salt-active" Corticoids as Reflected in the Concentrations of Sodium and Chloride of Thermal Sweat, J. Clin. Endocrinol. **10:** 12-23, 1950.
13. Conn, J. W.: Primary Aldosteronism. A New Clinical Syndrome, J. Lab. & Clin. Med. **45:** 6-17, 1955.
14. Conn, J. W.: Primary Aldosteronism, J. Lab. & Clin. Med. **45:** 661-664, 1955.
15. Lerner, A. B., Shizume, K., and Bunding, I.: The Mechanism of Endocrine Control of Melanin Pigmentation, J. Clin. Endocrinol. **14:** 1463-1490, 1954.
16. Shizume, K., and Lerner, A. B.: The Determination of the Melanocyte-stimulating Hormone in Urine and Blood, J. Clin. Endocrinol. **14:** 1491-1510, 1954.
17. Lerner, A. B., and Takahashi, Y.: To be reported in detail as part of a systematic study of factors which influence blood and urine levels of melanocyte-stimulating hormone.

STUDIES ON THE EFFECT OF ETHYLENEDIAMINETETRAACETIC ACID IN HYPERCALCEMIA

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HYPERCALCEMIA is frequently encountered in patients with malignant tumors affecting the skeleton. The early recognition of this serious complication may prevent the development of irreversible renal damage.[1] This investigation was initiated in order to study the value of sodium ethylenediaminetetraacetate (Na-EDTA) in the therapy of hypercalcemia. The following considerations led to the selection of this agent: it forms a strong calcium chelate in the physiologic pH range, it is not metabolized, and it is promptly excreted by the kidney.[2]

Previous studies have shown that the slow intravenous administration of Na-EDTA did not lower the serum calcium in normocalcemic patients, whereas the urinary calcium was significantly elevated on the day of the infusion.[3] This indicated that the calcium bound in the plasma was rapidly replaced by calcium from another source, most likely from bone, and that the bound calcium was readily excreted in the urine. It was assumed that this compensatory release of bone calcium may not occur in patients with elevated serum calcium if Na-EDTA is given in a dose which lowers the serum calcium just to but not below the normal range.

Holland and associates[4] have reported considerable lowering of the serum calcium of hypercalcemic patients by rapid infusions of fairly large doses of Na-EDTA; the effect upon urinary calcium excretion was not reported. Dudley and associates[5] have recently shown that repeated slow infusions of large doses of Na-EDTA have temporarily decreased the serum calcium of two hypercalcemic patients. A marked increase of the calciuria was noted on some of the days of the Na-EDTA infusions.

MATERIALS AND METHODS

Eight patients with hypercalcemia due to metastatic bone disease were studied. They were maintained on a low calcium intake containing approximately 150 mg. calcium and 600 mg. phosphorus per day. Na-EDTA† was given intravenously, in the fasting state, in

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†Na-EDTA was obtained as a 20 per cent solution in ampules from the Riker Laboratories, Inc., Los Angeles. We thank Dr. Norman W. Karr for supplying this product.

amounts depending on the serum calcium level. In 5 patients (Cases 1, 2, 3, 4, and 7), the chelating agent was injected in 10 to 15 minutes, with an infusion of 200 ml. of 5 per cent glucose in water. In three patients (Cases 5, 6, and 8), Na-EDTA was administered intravenously in 4 hours with an infusion of 500 ml. of 5 per cent glucose in water. The dose was estimated in the following manner: Since 1 Gm. Na-EDTA binds 0.108 Gm. Ca^{++}, an amount of Na-EDTA was injected which would bind the excess calcium estimated to be present in the extracellular space of the patient. For instance, if the serum calcium was 15 mg. per cent and the extracellular fluid was estimated to be 10 L., less than 5 Gm. Na-EDTA was infused. Urine was collected several days prior to the Na-EDTA infusion, on the day of the infusion, and for one or several days thereafter. These collections were made in 4-, 4-, and 16-hour specimens in all but one patient receiving the rapid infusions; 24-hour specimens were collected of patients receiving the slow infusions. Blood samples were drawn prior to the infusion, at the end of it, and 1, 4, 8, and 24 hours after the start of the rapid infusion and 4, 8, and 24 hours after the start of the slow infusion.

The serum calcium was determined by the Kramer-Tisdall method[6] (oxalate precipitable calcium) and on samples which were ashed at acid pH prior to oxalate precipitation (total serum calcium). The urinary calcium was determined on acidified specimens ashed in the muffle furnace.[7] The calcium which could be bound in the urine by the amount of EDTA present therein was estimated in the following manner: 10 ml. NH_4OH (25 per cent) was added to 50 ml. urine, and the sample was allowed to stand for 10 to 15 minutes. The precipitate was separated from the supernatant by centrifugation. The supernatant was decanted, and concentrated H_2SO_4 was added drop by drop to the supernatant until the reaction became acid. This specimen was then evaporated in the drying oven and ashed in the muffle furnace. The ash was dissolved and calcium was determined.[7] This calcium represents the fraction bound to EDTA.

RESULTS

The diagnoses, the duration of hypercalcemia, the serum calcium levels, the amount of Na-EDTA infused and the speed of its administration, and the effect upon serum calcium are listed in Table I.

In order to illustrate the changes of serum and urinary calcium induced in a normocalcemic subject, the effect of 2 Gm. Na-EDTA infused in 40 minutes to a 26-year-old *normal,* ambulatory woman is shown in Fig. 1. There was only a slight change of the oxalate precipitable serum calcium; it was lowest at the end of the infusion, 9.2 mg. per cent, and rose rather rapidly to the preinfusion level. A marked rise in total serum calcium occurred, the highest amount being 13.8 mg. per cent at the time when the oxalate precipitable calcium was 9.6 mg. per cent. The bound calcium is represented by the dotted area between the two lines. The urinary calcium rose considerably from 44 mg. per twenty-four hours to 208 mg. on the day of the study; in other words, an excess of 168 mg., corresponding to 75 per cent of the binding power of the administered dose of Na-EDTA, was excreted.

The effects of 4 Gm. of Na-EDTA infused on 3 successive days in 15, 15, and 8 minutes to a *hypercalcemic* patient are shown in Table II and Fig. 2. This 58-year-old man had carcinoma of the kidney, with widespread osteolytic metastases. At the time of the study, he was in hypercalcemia for a period of 3 weeks, the only clinical manifestation of this syndrome being lethargy. The serum phosphorus was 3.3 mg. per cent; the alkaline phosphatase, 4.1 Bodansky units; the blood urea nitrogen, 25 mg. per cent; and the creatinine, 2.3 mg. per cent. The urinary output was adequate, 1,300 to 1,400 ml. per twenty-four

TABLE I. EFFECT OF NA-EDTA UPON SERUM CALCIUM LEVELS OF HYPERCALCEMIC PATIENTS

TABLE I. EFFECT OF Na-EDTA UPON SERUM CALCIUM LEVELS OF HYPERCALCEMIC PATIENTS

CASE NO.	AGE	SEX	DIAGNOSIS	BUN MG. %	DURATION OF HYPERCALCEMIA	Na-EDTA NO.	Na-EDTA GM.	INFUSION DURATION	SERUM CALCIUM MG. % 0 MIN.	15 MIN.	1 HR.	4 HR.	8 HR.	24 HR.	REMARKS
1	48	F	Carcinoma of breast with widespread osteolytic metastases.	30	5 months	1	7	15 min.	17.8	11.3	–	15.5	15.8	–	In coma prior to infusion. Became alert during infusion but relapsed. Infusion 2 was given 8 hours after infusion 1. Infusions 3 and 4 were given on subsequent days.
									18.2**	19.5**	–	19.3**	19.5**	–	
						2	7	15 min.	15.8	12.0	–	–	17.2	17.1	
									19.5**	21.0**	–	–	17.6**	18.5**	
						3	7	15 min.	17.1	12.3	–	–	15.2	18.2	
									18.5**	19.9**	–	–	18.5**	18.5**	
						4	7	15 min.	18.2	12.2	–	–	–	12.7	
									18.5**	19.5**	–	–	–	18.0**	
2	68	M	Multiple myeloma with diffuse bone involvement.	36	Unknown, at least 6 months	1	4	15 min.	14.0	10.4	–	13.6	–	14.4	Infusion 2 was given 4 weeks after infusion 1.
									–	–	–	–	–	–	
				35		2	4	15 min.	13.3	10.1	11.3	11.4	–	13.5	
									13.7**	13.3**	13.5**	14.4**	–	14.0**	
3	55	F	Carcinoma of breast with widespread osteolytic metastases.	32	1 month	1	4	15 min.	14.4	10.8	12.4	14.4	14.2	13.5	
									13.1**	13.9**	14.0**	14.5**	13.9**		
4	42	F	Carcinoma of breast with widespread osteolytic metastases.	27	1 month	1	5.1	10 min.	13.2	3.0	9.6*	–	12.2	–	Tetany during infusion, relieved by 40 c.c. of 10% calcium gluconate (360 mg. Ca^{++}) intravenously. Blood for serum calcium taken at 10 min. instead of 15 min. (3.0 mg. %).
									13.3**	19.7**	15.6**	–	13.7**	–	
5	52	M	Carcinoma of kidney with widespread osteolytic metastases.	16	3½ months	1	4	4 hours	12.7	–	–	10.1	10.7	11.9	
									11.2**	–	–	10.7**	–	11.0**	
6	53	M	Malignant lymphoma. Severe bone pain. No x-ray evidence of bone involvement.	44	1 month	1	6	4 hours	17.3	–	–	15.1	18.1	17.7	Infusion 2 was given 2 days after infusion 1.
									18.4**			18.2**	19.5**	20.2**	
						2	7	4 hours	17.2	–	–	14.5	14.9	15.5	
									17.1**	–	–	18.5**	18.4**	18.2**	

*15 minutes after 360 mg. Ca^{++} were given intravenously.
**Total calcium.
Case 7 is described in Table II and Fig. 2, Case 8 in Fig. 3.

hours, and there was no albuminuria. During and following each infusion, the lethargy changed to euphoria, which lasted for several hours so that the patient commented on this improvement. There were no untoward clinical or chemical changes attributable to EDTA; the prothrombin time was normal prior to and after the infusions, and there was no albuminuria. The oxalate precipitable serum calcium decreased from 14.5 to 12.8 mg. per cent, from 14.0 to 11.9 mg. per cent, and from 14.1 to 11.3 mg. per cent at the end of the infusions on the three days, respectively. It had further decreased 45 minutes later and was still lower than the preinfusion level 8 hours after the infusion

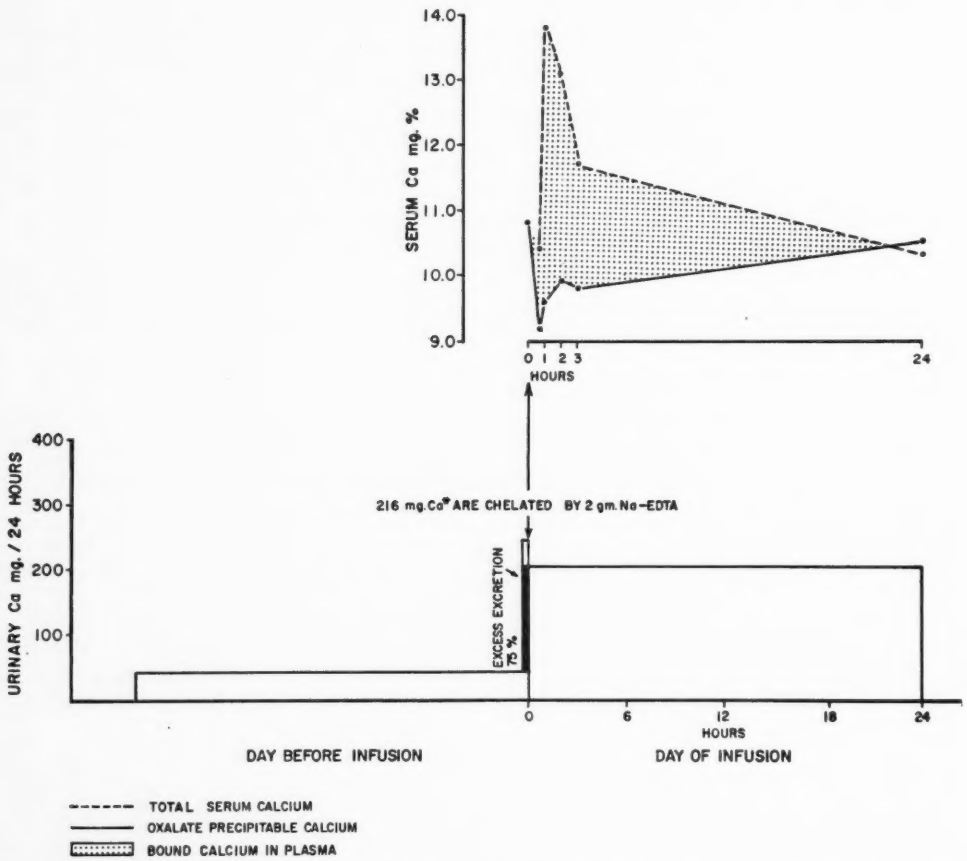

Fig. 1.—Effect of intravenous Na-EDTA upon serum and urinary calcium of a normocalcemic subject.

in studies 1 and 3, whereas it had returned to the preinfusion level by the eighth hour in study 2. In contrast to the normocalcemic patient, the total serum calcium did not rise. It showed slight fluctuations in studies 2 and 3 and was 1.2 mg. per cent lower than the preinfusion level 4 hours after the infusion in study 1. The 24-hour urinary calcium excretion on the day prior to the administration of Na-EDTA was 372 mg. On the 3 days of the in-

fusions, it was 482, 494 and 510 mg. Therefore, the excess urinary calcium excreted on the 3 days of the Na-EDTA infusion was 110, 122, and 138 mg., corresponding to 26, 28, and 30 per cent of the binding power of the chelating agent, respectively. The EDTA which was excreted was not determined in the first two collections but only in the 16-hour specimens of the 3 studies. The calcium binding power corresponded to approximately half the total calcium excreted in these specimens: 160 versus 328 mg., 148 versus 326 mg., and 156 versus 342 mg. However, the amount of bound calcium contained in the

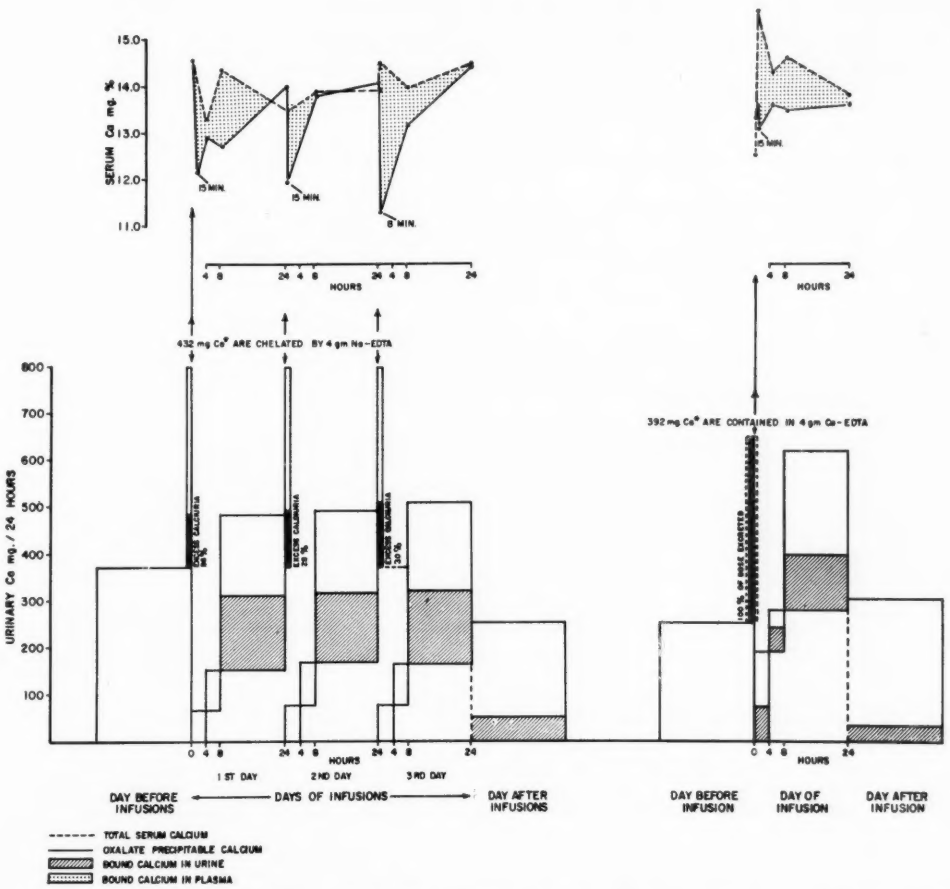

Fig. 2.—Effects of rapidly infused Na-EDTA and Ca-EDTA upon the serum and urinary calcium of a hypercalcemic patient. Ca-EDTA was administered 11 days after the third infusion of Na-EDTA (Case 7. See also Table II).

16-hour specimen was already higher than the total 24-hour excess mentioned above. Therefore, a significant fraction of the urinary calcium of the experimental day must have been excreted also in the form of calcium chelate. The 24-hour urine of the day after the infusion contained an amount of EDTA equivalent to 50 mg. calcium. The urinary calcium of this day was lower than that of the day prior to the infusion.

In order to test whether the comparatively low excess excretion was due to the inability of this patient to excrete the calcium chelate, 4 Gm. of Ca-EDTA was injected into the same patient 11 days after the last Na-EDTA study. The results are included in Table II and Fig. 2. The oxalate precipitable serum calcium remained essentially unchanged, whereas the total calcium had increased considerably by the end of the infusion, remained elevated for 8 hours, and approached the preinfusion level 24 hours later. The 24-hour urinary calcium excretion of the day of the Ca-EDTA infusion, determined on 4-, 4-, and 16-hour collections, was considerably increased, namely, from 256 mg. to 619 mg. The excess excretion was 363 mg., and the calcium content of the infused Ca-EDTA was 392 mg. A small excess urinary calcium was also present on the subsequent day. The EDTA excretion determined in all specimens was only 72 per cent of the theoretical, binding 284 mg. calcium.

TABLE II. EFFECT OF INTRAVENOUS NA-EDTA AND CA-EDTA UPON SERUM AND URINARY CALCIUM

EDTA INFUSION			SERUM CALCIUM – mg %						URINARY CALCIUM – mg. /24 hrs.					
			DAY OF INFUSION					24 HRS. POST I.V.	DAY BEFORE INFUSION	DAY OF INFUSION				DAY AFTER INFUSION
TYPE	GRAMS	DURATION	0 MIN.	15 MIN.	1 HOUR	4 HOURS	8 HOURS			4 HOURS	4 HOURS	16 HOURS	TOTAL	
NA-EDTA	4	15 minutes	14.5	12.8	12.3	12.9	12.7	14.0	372	69	85	328	482	—
			14.5	14.4	13.8	13.3	13.5	13.5	—	—	—	160		
NA-EDTA	4	15 minutes	14.0	11.9	—	12.9	13.8	14.1	—	77	91	326	494	—
			13.5	13.5	—	—	13.9	13.9	—	—	—	148	—	—
NA-EDTA	4	8 minutes	14.1	11.3†	—	12.3	13.2	14.4	—	79	89	342	510	257
			13.9	14.5	—	14.3	14.0	14.5	—	—	—	238	—	51
AFTER 11 DAYS														
CA-EDTA	4	15 minutes	14.3	14.6	14.1	14.6	14.5	14.6	256	191	90	338	619	308
			13.5	15.6	16.1	15.3	15.6	14.8	—	78	49	120	247	37

NA-EDTA INFUSIONS ON 3 CONSECUTIVE DAYS

† SERUM WAS DRAWN AFTER 8-MINUTE INFUSION

TOTAL SERUM CALCIUM

BOUND URINARY CALCIUM

Case 7. See also Fig. 2.

The changes of the serum and of the urinary calcium observed in this patient were similar to those of the remaining 4 patients, who received a total of 8 infusions of Na-EDTA. The increase of the urinary calcium on the day of the infusion, over and above that of the preinfusion day, was considerably less than in normocalcemic patients receiving a comparable dose of Na-EDTA. In some of these cases, no excess was noted.

Three patients (Cases 5, 6 and 8) received intravenously, in 4 hours, from 4 to 7 Gm. Na-EDTA in 500 ml. of 5 per cent glucose in water. All 3 patients showed lowering of the oxalate precipitable serum calcium. However, this improvement was only transient except in Case 8, which is described in greater detail.

CASE 8.—A 48-year-old woman had a radical mastectomy for carcinoma of the breast which involved the axillary lymph nodes; she had received postoperative local radio-therapy and was well for 5 months. She then developed increasing back pain suggestive of skeletal metastases, which became evident on roentgenograms 4 months later. Drowsiness, constipation, and vomiting, of several weeks' duration, followed. The patient was found to be in hypercalcemia, which ranged from 16.7 to 18.6 mg. per cent. The clinical course is illustrated schematically in the lower part of Fig. 3.

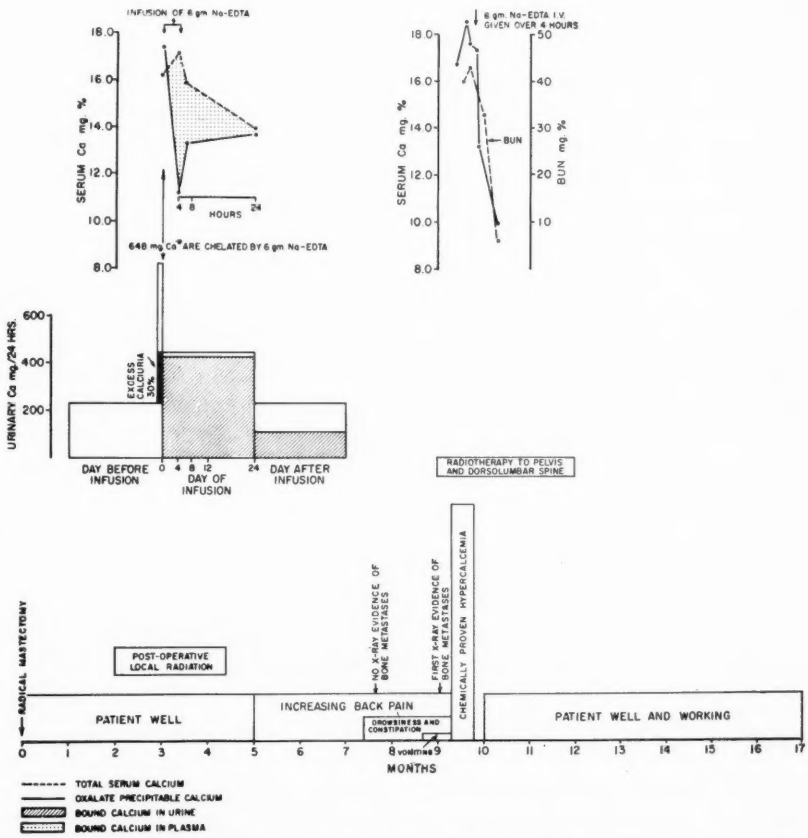

Fig. 3.—Effect of a single intravenous dose of Na-EDTA upon the serum calcium and urinary calcium of a hypercalcemic patient (Case 8).

The patient received a single dose of 6 Gm. Na-EDTA in 4 hours. The rather precipitous fall of the serum calcium level and of the blood urea nitrogen, which followed the administration of this agent, is shown in the center of the upper half of Fig. 3; the measurements of the serum and urinary calcium on the day of the Na-EDTA infusion are shown in the insert, left upper half of this figure. The oxalate precipitable serum calcium prior to the infusion was 17.4 mg. per cent; it decreased to 11.4 mg. per cent by the end of the infusion, and it was 13.2 mg. per cent the next day. The total serum calcium was increased

only slightly by the end of the infusion, and it decreased progressively by 24 hours. The dotted area between the two lines represents the bound calcium. The excess urinary calcium excretion on the day of the infusion was only 200 mg. or 30 per cent of the binding power of the injected Na-EDTA. However, all the calcium which was excreted on this day was chelated, since the EDTA which was present in this 24-hour specimen bound 426 mg. calcium. An additional 110 mg. "bound" calcium was excreted on the day after the infusion. The patient improved and became ambulatory. The serum calcium remained in the normal range and the patient returned to work. Ten months later, another episode of hypercalcemia developed and she received two more doses of 5 and 6.5 Gm. Na-EDTA; the oxalate precipitable serum calcium declined at the end of the infusions from 17.4 to 10.8 mg. per cent and from 14.7 to 11.6 mg. per cent, respectively. However, in contrast to the favorable response to the first dose, the improvement was not sustained at this time.

SIDE REACTIONS

Since 1951, 35 patients have received Na-EDTA and/or Ca-EDTA; the dose, the speed of its infusion, and the type of compound administered to 8 patients referred to in this communication are listed in Tables I and II and Fig. 3. The details pertaining to 11 patients were reported previously.[3] Fourteen of the remaining 16 patients received EDTA (2 to 4 Gm. of Na-EDTA or Ca-EDTA) for 1 to 6 days for investigative purposes, one for the treatment of Wilson's disease and another for chronic lead poisoning. This last patient received the largest total dose: 3 Gm. Ca-EDTA on 12 consecutive days, and 6 days later, 4 Gm. Na-EDTA on 6 consecutive days.

All patients were carefully observed during and following the injections of the chelating agent. Pulse, blood pressure, respiratory rate, blood counts, urinalysis, prothrombin time, bleeding time, and clotting time were frequently determined in most of these patients. A slight hypotension was noted during and a few hours subsequent to the administration of EDTA in most patients.[3] A transient slight albuminuria was found in one instance: in the above mentioned patient with chronic lead poisoning. Oliguria occurred in one patient (Case 1, Table I); she was in the terminal stage of her illness and was in hypercalcemia for 5 months; she received a large dose of Na-EDTA, 28 Gm. in 3 days, and expired 2 days later. Neither bleeding manifestations, prolongation of prothrombin time, nor changes in clotting mechanism were found.

Hypocalcemia was observed in one patient (Case 4, Table I). This occurred because the dose of EDTA was calculated on the basis of a serum calcium determination performed 2 days previously which was higher than that on the day of infusion: 15.6 mg. versus 13.2 mg. per cent. Also the estimate of the extracellular fluid volume was in error and too high. When 5.1 Gm. Na-EDTA was injected in 10 minutes and tetany occurred, the infusion was immediately discontinued, blood was drawn for calcium determination, and calcium gluconate was injected. The tetany was immediately relieved. The oxalate precipitable serum calcium analyzed in the blood which was drawn at the time of tetany was 3.0 mg. per cent, and the total serum calcium was 19.7 per cent. Therefore, the chelated calcium was 16.7 mg. per cent, corresponding to a total of 336 mg. in the plasma volume of 2010 ml. The elevation of the total calcium from 13.2 to 19.7 mg. per cent illustrated the speed with which calcium was drawn into the vascular compartment to replace the bound calcium therein.

TABLE III. POST-MORTEM FINDINGS OF HYPERCALCEMIC PATIENTS WHO RECEIVED Na-EDTA INTRAVENOUSLY

CASE NO.	AGE	SEX	DIAGNOSIS	EDTA					TIME INTERVAL BETWEEN LAST EDTA ADMINISTRATION AND DEATH	POST-MORTEM FINDINGS POSSIBLY ATTRIBUTABLE TO EDTA
				TYPE	GM./DAY	SPEED OF ADMINISTRATION	NO. DAYS	TOTAL GM.		
1	77	F	Carcinoma of breast with osteolytic metastases	Na	5	4 hours	1	5	7 days	None
2	42	F	Carcinoma of breast with liver metastases	Ca	4	6 hours	3	12		None
				Na	4	6 hours	3	12	4 months	
3	68	M	Carcinoma of prostate with osteoblastic metastases	Ca	4	4 hours	1	4		None
				Na	4	4 hours	1	4		
				Ca	4	4 hours	3	12		
				Na	4	4 hours	3	12	6 weeks	
4	64	F	Carcinoma of breast with osteolytic metastases	Na	2	6 hours	6	12	6 months	None
5	63	F	Malignant lymphoma	Na	4	4 hours	1	4	3 months	None
6	46	F	Carcinoma of breast with osteolytic metastases	Na	4	4 hours	3	12	8 months	None
7*	53	M	Malignant lymphoma	Na	6	4 hours	1	6		None
				Na	7	4 hours	1	7	2 days	
8†	42	F	Carcinoma of breast with osteolytic metastases	Na	5.1	10 min.	1	5.1	3 weeks	None
9‡	48	F	Carcinoma of breast with osteolytic metastases	Na	14	30 min.	1	14		Considerable vacuolization and occasional necrosis of tubular epithelium, granular changes of cytoplasm. Collecting tubules normal
				Na	7	15 min.	1	7		
				Na	7	15 min.	1	7	2 days	
10	60	F	Carcinoma of breast with osteolytic metastases	Ca	4	4 hours	3	12	2 weeks	None
11	62	M	Carcinoma of lung	Ca	4	4 hours	1	4	3 months	None

*See Case 6, Table I.
†See Case 4, Table I.
‡See Text and Case 1, Table I.

Autopsy was performed in 11 patients; the diagnoses, the dose, type of compound, and the time interval which elapsed between the administration of the chelating agent and the death of the patient are listed in Table III.

DISCUSSION

It has been previously shown that the serum calcium levels of animals can be significantly lowered, even to levels of tetany, when large amounts of Na-EDTA are injected rapidly.[8] Similar observations were also made in hypercalcemic patients.[4] Studies performed in this laboratory have shown that the slow infusion of Na-EDTA did not lower the serum calcium level of normocalcemic patients but caused a marked excess urinary calcium excretion.[3] The maintenance of the serum calcium level and the excess calciuria observed in normals were attributed to the immediate replenishment of calcium bound in plasma by calcium from the body stores, most likely from the skeleton, the calcium chelate being excreted in the urine. It was assumed that excess calcium will be released from the skeleton only if the serum level is lowered below the normocalcemic range. The observations made on 8 hypercalcemic patients seem to support this theory: the oxalate precipitable serum calcium was decreased to levels which were not below the normal range,* whereas the total calcium remained unchanged. A large amount of bound calcium was present in the plasma which declined with time as the calcium chelate was excreted in the urine. How could this small but definite excess calciuria observed in most hypercalcemic patients be reconciled with the theory that calcium present in excess of the normal range in the extracellular compartment could be bound without causing calcium release from the body store? It is possible that transient hypocalcemia was induced during the period of injection of Na-EDTA, although this was not detected in the blood sample drawn for analysis. Furthermore, it is possible that *any* lowering of plasma calcium levels elicits compensatory forces which, however, are weaker in hypercalcemia than those which safeguard the maintenance of normal calcium levels. The study of Patient 7 (Table II and Fig. 2) indicates that the smallness of the increment in calciuria is not due to the inability of hypercalcemic patients to excrete the calcium through the kidney.

Na-EDTA when injected intravenously binds calcium in the plasma instantaneously. It is therefore likely that, as the calcium is bound in the plasma, calcium ions enter the vascular compartment from the interstitial fluid compartment, and thereby a new equilibrium for the ionized calcium is established. The speed with which this occurs was illustrated in the study of the patient in whom tetany occurred: at that moment the oxalate precipitable calcium fell, whereas the total calcium increased considerably over the preinfusion level. This method of lowering the ionized plasma calcium levels in normal subjects or in hypercalcemic patients seems to provide a potent tool for the

*In Case 4 (Table I), hypocalcemia occurred, as described in the section on side reactions.

investigation of forces to maintain homeostasis: the speed with which it is re-established may be a highly sensitive measure of the available calcium pool and of the metabolic state of skeleton.

The calcium chelate diffuses freely from the intravascular compartment into the interstitial space and into the body cells, with the exception of red blood corpuscles and of the cerebrospinal fluid.[2] It then re-enters the vascular compartment[10] and is excreted. The presence of large amounts of bound calcium in the plasma indicates that the elimination of the calcium chelate is much slower than the binding. Foreman[2a and 2b] has shown that approximately 98 per cent of the C^{14}-labeled EDTA was excreted in the urine of animals and of normal human subjects within 24 hours. In a previous study, we attempted to gauge the EDTA excretion indirectly through the determination of the 24-hour excess calciuria which was found to be in average only 75 per cent of the theory. This discrepancy between Foreman's data and ours could be due to differences in the experimental subjects which were used. It is more likely, however, that these differences are due to the liberation of calcium from the Ca-EDTA, with the prompt excretion of the EDTA and the retention of calcium in the kidneys. Attempts to clarify this problem by the simultaneous determination of calcium and of EDTA have failed (see experimental section), probably because of the inaccuracy of the method to determine EDTA in urine. It seems that the suspected dissociation of the Ca-EDTA complex and the retention of calcium without EDTA could be proved by using better techniques to measure the urinary excretion of EDTA: for instance, with labeled EDTA.

From the clinical point of view, the chelation of excess ionized calcium in the circulation and the excretion of calcium as the chelate in place of free calcium are desirable in order to decrease hypercalcemic toxicity and to prevent the development of nephrocalcinosis. However, Na-EDTA decreases only temporarily the serum calcium in hypercalcemic patients with metastatic bone disease as the bone breakdown caused by the tumor continues. Favorable clinical and chemical results were obtained in only one of the eight hypercalcemic patients with metastatic bone disease. This may have been coincidental with the administration of Na-EDTA and due to a spontaneous remission of osteolytic activity of the tumor in the bone.[9]

Although the benefits were only transient in 7 of the 8 patients, the judicious use of EDTA to alleviate hypercalcemic toxicity is justified in cases in whom medical, radiotherapeutic, or surgical measures are contemplated: for instance, prior to the removal of a parathyroid adenoma. The usefulness of EDTA will greatly depend upon the frequency and severity of toxicity side reactions. Holland and associates[4] have reported hydropic degeneration of the tubular epithelium in 2 hypercalcemic patients who received 10 and 9.5 Gm. Na-EDTA in 54 and 60 minutes, respectively. Both patients were in uremia and died 4 days after the administration of Na-EDTA. Similar extensive pathologic changes of the kidneys were recently described by Dudley and associates[5] in 2 patients. One of these was in the terminal stage of cancer of the breast with osteolytic metastases: she received a total of 145 Gm. Na-EDTA in 13 days and expired 1 day later. The second patient was a 4½-year-old girl who had hyper-

calcemia due to vitamin D intoxication; she received 21 Gm. Na-EDTA in 10 days and died 8 days later. Hemorrhagic manifestations and engorgement of the reticuloendothelial cells with coarse eosinophilic granules were noted in both patients. As described in section "Side Reactions" no hemorrhagic manifestations were noted in any of the 35 cases of our series. Only in 1 of 11 postmortem examinations were changes noted in the kidney similar to those described by Holland and associates and Dudley and co-workers. This patient was comatose and terminal; she was in severe hypercalcemia when she received a large dose of Na-EDTA in a short period of time. She expired 2 days after the fourth dose of Na-EDTA.

It may be assumed that the toxicity of Na-EDTA is similar to that of Ca-EDTA except for the manifestations of calcium withdrawal caused by the former. The problems related to the toxicity of Ca-EDTA, however, are of greater practical importance since Ca-EDTA is currently being used rather widely in the treatment of lead poisoning and is also being considered for the forced removal of radioactive fission products entering the human body. To our knowledge, no reports are available in medical literature which refer to severe toxic manifestations following the prolonged use of Ca-EDTA. No major untoward side effects were noted in our series. However, we have used Ca-EDTA principally for investigative purposes in relatively small doses given over a short period of time. The observations of Holland and of Dudley suggest that extreme caution should also be exercised when large doses of Ca-EDTA are being used for therapy over a long period of time.

SUMMARY

1. The chelating agent sodium ethylenediaminetetraacetate was administered to 8 hypercalcemic patients. In each of these patients, the serum calcium was temporarily lowered as the excess calcium was chelated; the calcium chelate was excreted by the kidneys.

2. In normocalcemic subjects, the chelation of the serum calcium is promptly followed by release of calcium from body stores to maintain the ionized calcium at normal levels. The total calcium increases because of this influx of calcium into the circulation and returns to normal levels as the calcium chelate is excreted. The excess calciuria reflects the magnitude of the calcium influx.

3. The responses to the administration of the chelating agent, i.e., the speed of restoration of the serum calcium level and the excess calciuria, seem to be sensitive indicators of the state of calcium metabolism.

4. The therapeutic usefulness of Na-EDTA in the treatment of hypercalcemia and of Ca-EDTA in the treatment of metal poisoning are discussed in conjunction with the side reactions attributed to these agents.

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REFERENCES

1. Swyer, A. J., Berger, J. S., Gordon, H. M., and Laszlo, D.: Hypercalcemia in Osteolytic Metastatic Cancer of the Breast, Am. J. Med. 8: 724, 1950.

2a. Foreman, H., Vier, M., and Magee, M.: The Metabolism of C^{14}-labeled Ethylenediamine Tetra-acetic Acid in the Rat, J. Biol. Chem. **203**: 1045, 1953.
2b. Foreman, H., and Trujillo, T. T.: The Metabolism of C^{14}-labeled Ethylenediamine Tetra-Acetic Acid in Human Beings, J. LAB. & CLIN. MED. **43**: 566, 1954.
3. Spencer, H., Vankinscott, V., Lewin, I., and Laszlo, D.: Removal of Calcium in Man by Ethylenediamine Tetra-acetic Acid, A Metabolic Study, J. Clin. Invest. **31**: 1023, 1952.
4. Holland, J. F., Danielson, E., and Sahagian-Edwards A.: Use of Ethylenediamine Tetra-acetic Acid in Hypercalcemic Patients, Proc. Soc. Exper. Biol. & Med. **84**: 359, 1953.
5. Dudley, H. R., Ritchie, A. C., Schilling, A., and Baker, W. H.: Pathologic Changes Associated With the Use of Sodium Ethylene Diamine Tetra-Acetate in the Treatment of Hypercalcemia, New England J. Med. **252**: 331, 1955.
6. Clark, E. P., and Collip, J. B.: A Study of the Tisdall Method for the Determination of Blood Serum Calcium With a Suggested Modification, J. Biol. Chem. **63**: 461, 1925.
7. Shohl, T. A., and Pedley, F. G.: A Rapid and Accurate Method for Calcium in Urine, J. Biol. Chem. **50**: 537, 1922.
8. Popovici, A., Geschickter, C. F., Reinovsky, A., and Rubin M.: Experimental Control of Serum Levels in Vivo, Proc. Soc. Exper. Biol. & Med. **74**: 415, 1950.
9. Laszlo, D., Schulman, C. A., Bellin, J., Gottesman, E. D., and Schilling, A.: Mineral and Protein Metabolism in Osteolytic Metastases, J. A. M. A. **148**: 1027, 1952.
10. Hart, H. E., Greenberg, J., Lewin, R., Spencer, H., Stern, K. G., and Laszlo, D.: Metabolism of Lanthanum and Yttrium Chelates, J. LAB. & CLIN. MED. **46**: 182-192, 1955.

BLOOD PYRUVIC ACID, SERUM GAMMA GLOBULIN, AND OTHER TESTS OF HEPATIC FUNCTION

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SOME fifty-seven years after Bernard[1, 2] demonstrated the vital importance of the liver in carbohydrate metabolism, Kostytschev,[3] and Neuberg and Kerb[4] discovered that pyruvic acid was produced as an intermediate in the fermentation of sugar. As the commanding role of this keto-acid in carbohydrate metabolism has since been amply confirmed,[5-7] it is rather surprising to find that very few studies have been carried out on blood pyruvate levels in hepatic disorders.

During a study of carbohydrate metabolism in patients ill with liver disease,[8] the basal levels of blood pyruvate were measured, as well as a number of tests made of hepatic function. Although the blood pyruvate level was found to be high in only two-thirds of the patients ill with liver disease, a considerable degree of correlation was found to exist between the basal levels of pyruvic acid in the blood and two of the tests of hepatic function used in this study. These observations are, therefore, reported in this paper.

MATERIAL AND METHODS

Subjects.—Thirty-nine patients were studied in three hospitals. Of these, twenty-five had definite clinical and laboratory evidence of hepatic disease; three were suspected of having hepatic disease; and the remainder were in the hospital for the medical care of some other ailment. Although none of the patients were ill with beriberi, the possibility of thiamine deficiency influencing the blood pyruvate level[9-11] was excluded by intramuscular injection of thiamine hydrochloride (100 mg.) into each patient for at least three days prior to the collection of blood.

All subjects were confined to bed except when they were allowed to go to the bathroom. They were not allowed any food after the midnight previous to the day of the test; otherwise the diet was not restricted.

Collection of Blood.—Before breakfast, 20 ml. of free flowing venous blood was collected. Immediately thereafter, 5 ml. of blood was ejected from the syringe into a reaction vessel containing 25 ml. of 10 per cent trichloracetic acid solution. The rest of the blood was run into a clean, dry test tube for separation of serum for other tests of hepatic function.

Laboratory Methods.—Blood pyruvic acid was determined essentially by the method of Friedemann and Haugen,[12] except that pyruvic-acid-hydrazone was extracted with two 5 ml. portions of ethyl acetate instead of by use of a single extraction. Spectrophotometric readings of the sodium hydroxide-treated hydrazone were made at 520 mμ.

The following tests of hepatic function were carried out in the serum of each patient: (a) serum bilirubin by the modified method of Ducci and Watson[13]; (b) serum cholinesterase by the method of Michel[14]; (c) serum gamma globulin by the method of de la Huerga and

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Popper[15]; (d) thymol turbidity by the method of Shank and Hoagland[16]; (e) thymol flocculation test as modified by Neefe and associate[17, 18]; (f) zinc sulfate turbidity test as described by Kunkel[19]; and (g) the cephalin flocculation test of Hanger.[20]

RESULTS

1. *Correlation Between Basal Blood Pyruvate Level and Tests of Hepatic Function in All Subjects.*—The basal levels of blood pyruvic acid and the results of the tests of liver function are shown in Table I. With these data, scatter diagrams were drawn, plotting the blood pyruvate levels against the results of each of the tests of hepatic function. On inspection of these diagrams, the basal blood pyruvate level was seen to correlate well with serum gamma globulin concentration and thymol turbidity units (Figs. 1 and 2). Some degree of negative correlation was also apparent when the levels of serum cholinesterase were plotted against the basal levels of blood pyruvate (Fig. 3). There did not seem to be any correlation between the blood pyruvate level and the level of total serum bilirubin (Fig. 4) or any of the other tests of hepatic function.

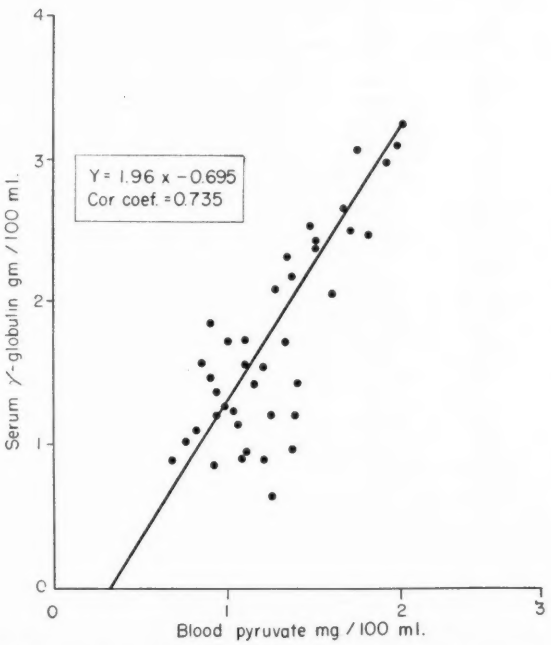

Fig. 1.—Serum gamma globulin and fasting blood pyruvate levels in 39 patients with and without liver disease. Note that there is a positive correlation, the correlation coefficient being 0.735.

Correlation coefficients were calculated[21] between the basal blood pyruvate level and the serum gamma globulin concentrations, thymol turbidity units, and the serum cholinesterase levels. As shown in Table II, these coefficients (the tests of hepatic function and the blood pyruvate levels since the values of "p" "r" values) were found to be statistically significant for each of the three were less than 0.001 in each case.

The regression equations and the standard errors of the estimations from the least squares' regression lines were also calculated[22] (Table II).

TABLE I. BASAL BLOOD PYRUVATE LEVELS AND TESTS OF HEPATIC FUNCTION IN 39 PATIENTS WITH AND WITHOUT LIVER DISEASE

PATIENT'S NO. AND NAME	FASTING BLOOD PYRUVATE (MG./100 ML.)	SERUM GAMMA-GLOBULIN (GM./100 ML.)	THYMOL TURBIDITY (UNITS)	SERUM CHOLINESTERASE (Δ pH/HR.)	SERUM BILIRUBIN (MG./100 ML.)	$ZnSO_4$ TURBIDITY (UNITS)	THYMOL FLOCCULATION (+UNITS)	CEPHALIN FLOCCULATION (+UNITS) 24 HR.	CEPHALIN FLOCCULATION (+UNITS) 48 HR.
2.* I. T.	1.27	2.08	8.50	0.88	0.95	14.90	4+	3+	3+
3.* L. B.	0.85	1.55	1.95	0.96	0.84	9.30	0	1+	2+
4.* B. C.	1.47	2.52	7.00	0.59	0.55	16.50	4+	4+	4+
5.* E. P.	1.50	2.42	7.40	0.87	0.74	17.00	2+	4+	4+
6.* D. B.	1.10	1.74	6.20	0.24	2.32	11.48	2+	4+	4+
7.* H. S.	1.50	2.36	5.00	0.29	1.56	10.90	1+	3+	4+
8.* H. W.	1.20	1.54	8.20	0.35	29.80	9.08	2+	3+	3+
9.* P. D.	1.60	2.04	6.50	0.25	2.63	12.30	1+	3+	4+
10.* T. Y.	1.34	2.30	6.00	0.55	2.13	14.12	0	3+	4+
12. C. S.	0.82	1.10	1.11	0.92	1.06	5.31	0	1+	1+
13. F. J.	1.02	1.22	1.73	0.54	0.66	7.42	0	1+	2+
14. L. D.	1.10	1.55	2.41	0.79	0.36	10.05	0	1+	1+
15. E. G.	0.68	0.89	1.52	1.26	0.47	5.52	0	0	1+
16.* T. M.	1.66	2.65	9.70	—	3.84	15.54	2+	3+	3+
17.* K. R.	1.90	2.96	11.90	0.23	2.50	14.52	3+	3+	3+
18. J. M.	1.37	0.96	1.11	—	0.48	4.02	0	0	0
19. R. M.	1.15	1.42	2.30	—	1.32	9.00	0	0	1+
20.* B. G.	1.80	5.42	14.25	0.27	12.22	26.70	4+	4+	4+
21. R. B.	1.38	1.22	2.58	0.81	1.20	7.70	0	0	1+
22. M. M.	0.76	1.01	1.87	1.65	0.74	7.10	0	0	0
23. T. D.	1.08	0.90	2.12	0.74	0.52	5.60	0	0	0
24.* E. M.	1.36	2.17	6.82	0.37	32.60	6.90	Trace	0	0
25.* J. C.	1.74	3.06	13.10	0.38	1.85	19.78	3+	3+	4+
26. R. D.	1.00	1.71	4.55	0.75	—	14.90	Trace	Tr.	2+
27.* B. F.	0.90	1.46	6.68	0.55	2.60	10.65	3+	3+	4+
28. O. J.	1.33	1.71	1.30	1.20	0.73	7.48	0	1+	2+
29. A. L.	0.94	1.20	3.25	1.08	—	4.75	0	0	1+
30. M. J.	1.20	0.89	4.20	1.02	—	2.68	0	0	0
31.* V. H.	0.98	1.27	2.80	0.65	1.82	6.36	0	0	1+
32.* R. T.	0.90	1.84	6.36	0.77	1.73	11.30	4+	3+	4+
33.* M. K.	1.40	1.43	8.90	0.68	11.34	8.48	2+	0	1+
34. D. R.	0.92	0.85	1.48	0.39	0.30	2.50	0	0	0
35.* E. M.	1.97	3.09	11.76	0.59	5.30	21.53	3+	3+	4+
36.* S. H.	1.75	2.49	10.10	0.44	3.16	12.25	2+	3+	4+
37.* M. P.	1.80	2.45	10.82	0.18	2.30	16.20	3+	3+	4+
38.* G. F.	0.93	1.36	1.21	0.40	0.33	6.90	0	1+	2+
40.* M. M.	1.10	0.94	2.10	1.12	24.60	6.20	0	1+	1+
41.* T. C.	1.25	1.02	2.23	0.33	12.30	12.50	3+	2+	3+
42. M. M.	1.25	0.66	1.43	0.73	0.84	4.55	0	1+	1+

*Clinically evident liver disease.

2. *Correlation of Basal Pyruvate Level and Flocculation Tests Among Patients Ill With Liver Disease and Those Without Liver Disease.*—In Table III are recorded the relationships between pyruvate levels in the blood and the flocculation tests in the serum of thirty-nine patients.

All the patients with abnormal flocculation tests had liver disease. However, a blood pyruvate level of 1.1 mg. per 100 ml. or over was observed in several subjects who did not have any evidence of hepatic disease. This fact is demonstrated in Fig. 5, which shows the distribution patterns of the basal blood pyruvate levels in two of the groups of patients studied, a group of fifty-one patients* with no evidence of liver disease and twenty-four patients known to have hepatic dysfunction.

*Data on thirty-eight patients included in the group of fifty-one without hepatic disorders were kindly supplied by Dr. M. K. Horwitt, Elgin State Hospital, Elgin, Ill.

TABLE II. THE RESULTS OF MATHEMATICAL ANALYSES OF THE CORRELATION BETWEEN BASAL BLOOD PYRUVATE (X) AND THREE TESTS OF HEPATIC FUNCTION (Y)

X*	Y†	N‡	CORRELATION COEFFICIENT§ (r) AND VALUE OF p‖	REGRESSION EQUATION $Y = aX + b$¶ X IS ASSUMED CORRECT	STANDARD ERROR (SE) †† X IS ASSUMED CORRECT $SE = SY^{**}\sqrt{1-r^2}$
Basal pyruvate in blood	Serum gamma-globulin	39	r = 0.735 p = <0.001	Y = (1.96) X −0.695	± 0.612 Gm./100 ml.
	Thymol turbidity	39	r = 0.778 p = <0.001	Y = (8.84) X −5.82	± 2.411 units
	Serum cholinesterase	36	r = 0.550 p = <0.001	Y = 1.35 −(0.55) X	± 0.286 ΔpH/hr.

*X = Basal blood pyruvate level in mg. per 100 ml.
†Y = (i) Serum gamma-globulin in grams per 100 ml., or
(ii) Thymol turbidity in units, or
(iii) Serum cholinesterase in △pH per hour.
‡N = Number of subjects.
§r = Correlation coefficient.
‖p = Probability of occurrence by chance alone.
¶a and b = Constants in the regression equation.
**sY = Standard deviation of Y.
††SE = Standard error of estimate from the least squares' regression line, when X is assumed to be correct.

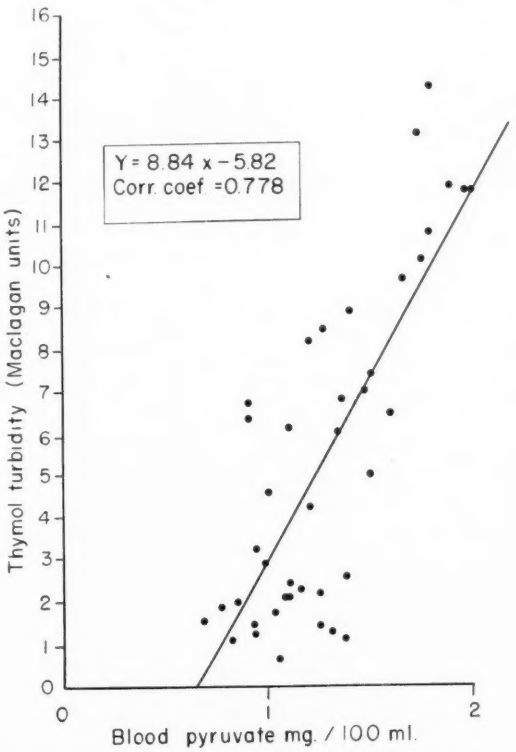

Fig. 2.—Blood pyruvate levels and thymol turbidity units in 39 patients with and without liver disease. Note that there is a positive correlation, the correlation coefficient being 0.778.

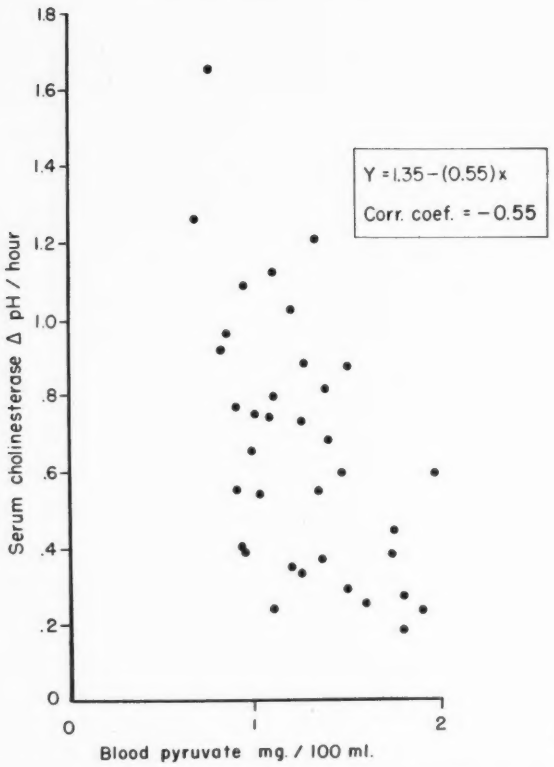

Fig. 3.—Blood pyruvate and serum cholinesterase levels in 36 patients with and without liver disease. Note that there is a negative correlation; the correlation coefficient equals -0.55.

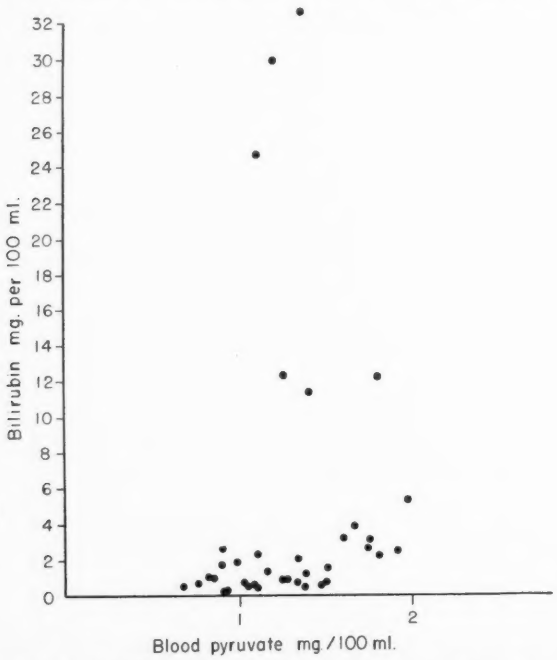

Fig. 4.—Blood pyruvate and bilirubin in 36 patients with and without liver disease. No correlation is apparent.

As can be seen in Fig. 5, a larger percentage of patients with liver disease had high basal blood pyruvate levels when compared with those of the control group. However, normal levels of blood pyruvate were found in about 30 per cent of patients with demonstrable hepatic insufficiency, while in 17 per cent of the control group abnormally high pyruvate levels were noted.

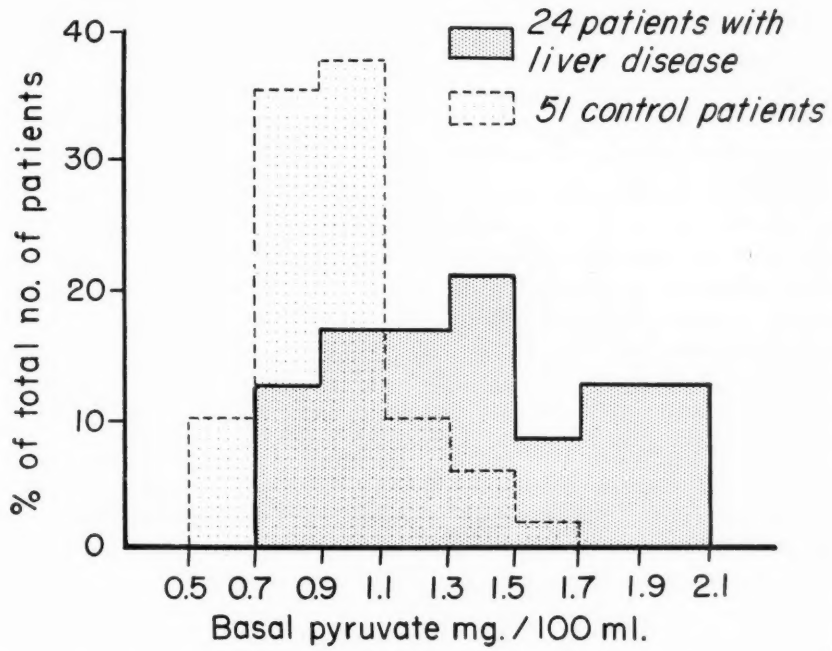

Fig. 5.—Distribution of basal pyruvate among 24 patients with liver disease (dark hatched areas) and 51 control patients free of liver disease (light hatched areas). Note considerable overlap in values.

TABLE III. ANALYSIS OF DATA FROM 39 SUBJECTS STUDIED

BASAL PYRUVIC ACID LEVEL	TOTAL NO. OF PATIENTS	NO. OF PATIENTS WITH ABNORMAL CEPHALIN FLOCCULATION TESTS	NO. OF PATIENTS WITH ABNORMAL THYMOL FLOCCULATION TESTS	NO. OF PATIENTS WITH BOTH FLOCCULATION TESTS ABNORMAL	NO. OF PATIENTS WITH LIVER DISEASE	NO. OF PATIENTS WITHOUT LIVER DISEASE
>1.1 mg./100 ml.	23	15	13	12	17	4
1.1 mg./100 ml. or less	16	8	3	3	7	8
Totals	39	23	16	15	24	12

The relation between the basal pyruvate levels and the flocculation tests is shown.

DISCUSSION

The mechanisms by which pyruvate may accumulate in blood may be summarized under three headings. These are:

1. *Increased Production of Pyruvate.*—This occurs during muscular exercise,[23] following meals (especially of carbohydrates)[9, 25] and following increased utilization of the reserve carbohydrates of the body, such as is seen in congestive

cardiac failure[24] and in thyrotoxicosis.[27] Increased utilization of reserve carbohydrates leading to raised pyruvic acid levels in the blood was also observed by us[28] when Glucagon was administered to a patient suffering from hyperinsulinism.

2. *Decreased Utilization of Pyruvate.*—Pyruvate is not readily metabolized in the presence of thiamine deficiency,[9-11] anoxia,[24, 26] and in severe disorders of the liver.[29, 30] In all these conditions it may accumulate in the blood.

3. *Impaired Rate of Removal of Pyruvate From the Circulation.*—In decompensated heart disease, impaired removal of pyruvate from the circulation has been observed.[24]

As noted previously, studies pertaining to the accumulation of pyruvate in blood in hepatic disorders have been reported by Amatuzio and associates.[29, 30] They measured pyruvic acid levels in the blood under basal conditions[29] as well as after intravenous administration of glucose[30] in normal people, in patients with liver diseases, in patients with diabetes, and in patients with emphysema. Thirty-six of their patients had liver disease. The basal levels of pyruvic acid were moderately elevated in patients with simple decompensated cirrhosis and returned to normal levels with adequate treatment. The highest levels of blood pyruvic acid were noted in patients with hepatic coma. Serial determinations showed an abrupt and progressive rise of the blood pyruvate level during the onset and progression of coma and a decline with improvement. A more recent study[30] by the same group of workers indicated that after intravenous glucose administration, glucose and pyruvate were utilized adequately in compensated cirrhosis of the liver. However, when patients in hepatic coma received glucose intravenously, large accumulations of pyruvate were found in their blood.

The present study confirms Amatuzio's observation that the basal blood pyruvate level is elevated in decompensated cirrhosis of the liver. In seventeen of twenty-four patients ill with liver disease, the basal blood pyruvate level was more than 1.1 mg. per 100 ml. Eleven of these seventeen patients were ill with advanced cirrhosis of the liver. Of the seven patients ill with liver disease who had normal blood pyruvate levels, one (No. 40, M. M.) had obstructive jaundice due to carcinoma of the ampulla of Vater, three (Nos. 27, 31, and 32) were suffering from viral hepatitis, and the rest (Nos. 3, 6, and 38) were ill with early cirrhosis of the liver.

From this analysis it is safe to conclude that the fasting blood pyruvic acid level is elevated in many patients with hepatic injury. This abnormality appears to be related to the severity of the hepatic disorder. Because abnormal levels of blood pyruvic acid were observed in only two-thirds of the patients ill with liver disease, the measurement of this metabolite in the blood cannot be regarded as a sensitive test of hepatic function.

In both healthy and diseased individuals, the basal blood pyruvic acid level varied directly with the concentration of serum gamma globulin. The thymol turbidity values, which depend largely upon the serum concentrations of gamma globulin,[31] also varied directly with the basal levels of blood pyruvate. This may lead one to speculate that pyruvic acid may be related in some way

or other to the synthesis or breakdown of serum gamma globulin. Unfortunately, the present knowledge of protein synthesis is in its infancy, and the role of pyruvic acid in this process is quite obscure.

Some degree of negative correlation was also observed between the level of serum cholinesterase and the basal level of blood pyruvate (Fig. 3). Although the probability that such a correlation occurred by chance alone is less than one in one thousand, no conclusion seems desirable at this time as the correlation coefficient ("r" = −0.55) was far from unity.

With regard to the flocculation tests, analysis of Table III shows that abnormal flocculation tests were found only in those who were ill with liver disease, and only a very few patients were observed to have normal flocculation tests in the presence of hepatic disorders. On the other hand, blood pyruvate levels were within normal range in five patients ill with liver disease, and a high blood pyruvate level was obtained in four patients without liver disease. This also indicated that the measurement of basal blood pyruvate level is less useful than the flocculation tests in assessing hepatic dysfunction.

SUMMARY

1. Measurement of basal blood pyruvate levels was compared with seven tests of hepatic function in thirty-nine patients.

2. High levels of pyruvate were noted in the fasting blood samples in two-thirds of the twenty-four patients ill with hepatic disease and in four of the fifteen patients without liver disease. This indicated that measuring the fasting blood levels of pyruvate is of limited value in assessing hepatic dysfunction in man.

3. In both healthy and diseased individuals, a high degree of positive correlation was observed between the serum concentrations of gamma globulin and the basal levels of blood pyruvate. The values of thymol turbidity, which depends upon the serum concentration of gamma globulin, were found to have a similar correlation, while a negative correlation of lesser magnitude was noted between the serum cholinesterase activity and the basal levels of blood pyruvate.

REFERENCES

1. Bernard, C.: Sur une nouvelle function du foie chez l'homme et les animaux, Comp. rend. Acad. Sc. **31**: 571, 1850.
2. Bernard, C.: Sur le micanisme de la formation du sucre dans le foie, Comp. rend. Acad. Sc. **41**: 461, 1855.
3. Kostytschev, S.: Ztschr. physiol. Chem. **79**: 130, 1912, As cited by Harden, A., in Alcoholic Fermentation, Ed. 3, London, 1923, Longmans, Green & Company, p. 97.
4. Neuberg, C., and Kerb, J.: Ztschr. Gärungsphysiol. **1**: 114, 1912, As cited by A. Harden in Alcoholic Fermentation, Ed. 3, London, 1923, Longmans, Green & Company, p. 97.
5. Nord, F. F., and Weiss, S.: Yeast and Mold Fermentation, in J. B. Sumner and K. Myrback edition of The Enzymes, New York, 1951, Academic Press.
6. Dickens, F.: Anerobic Glycolysis, Respiration and the Pasteur Effect, in J. B. Sumner and K. Myrback edition of The Enzymes, New York, 1951, Academic Press.
7. Elsden, S. R.: Bacterial Fermentation, in J. B. Sumner and K. Myrback edition of The Enzymes, New York, 1951, Academic Press.
8. Parida, R. K.: Carbohydrate Index in Assessing Hepatic Dysfunction, thesis submitted to the Graduate College of the University of Illinois, Chicago, 1955.
9. Horwitt, M. K., Liebert, E., Kreisler, O., and Wittmann, P.: Investigations of Human Requirements for B-complex Vitamins, Bull. Nat. Res. Council **116**: 1948.

10. Lu, G. D.: Studies on the Metabolism of Pyruvic Acid in Normal and Vitamin B_1 Deficient States, Biochem. J. 33: 249, 774, 778, 1939.
11. Platt, B. S., and Lu, G. D.: The Accumulation of Pyruvic Acid and Other Carboxyl Compounds in Beri-beri and the Effect of Vitamin B_1 Biochem. J. 33: 1525, 1939.
12. Friedemann, T. E., and Haugen, G. E.: Pyruvic Acid. II. The Determination of Keto-acids in Blood and Urine, J. Biol. Chem. 147: 415, 1943.
13. Ducci, H., and Watson, C. J.: The Quantitative Determination of Serum Bilirubin With Special Reference to the Prompt-reacting and the Chloroform Soluble Types, J. Lab. & Clin. Med. 30: 293, 1945.
14. Michel, H. O.: Electrometric Method for the Determination of Red Blood Cell and Plasma Cholinesterase Activity, Fed. Proc. 8: 229, 1949.
15. de la Huerga, J., and Popper, H.: Estimation of Serum-Gamma-Globulin Concentration by Turbidimetry, J. Lab. & Clin. Med. 35: 459, 1950.
16. Shank, R. E., and Hoagland, C. L.: A Modified Method for the Quantitative Determination of the Thymol Turbidity Reaction of the Serum, J. Biol. Chem. 162: 133, 1946.
17. Neefe, J. R.: Results of Hepatic Tests in Chronic Hepatitis Without Jaundice, Gastroenterology 7: 1, 1946.
18. Neefe, J. R., and Reinhold, J. G.: Laboratory Aids in the Diagnosis and Management of Infectious Hepatitis, Gastroenterology 7: 393, 1946.
19. Kunkel, H. G.: Estimation of Alterations of Serum Gamma-Globulin by a Turbidimetric Technique, Proc. Soc. Exper. Biol. & Med. 66: 217, 1947.
20. Hanger, F. M.: Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin-Cholesterol Emulsions, J. Clin. Invest. 18: 269, 1939.
21. Fisher, R. A.: Statistical Methods for Research Workers, New York, 1950, Hafner Publishing Company.
22. Mainland, D.: Elementary Medical Statistics, Philadelphia, 1952, W. B. Saunders Company.
23. Asmussen, E.: Pyruvate and Lactate Content of the Blood During and After Muscular Work, Acta Physiol. scandinav. 20: 125, 1950.
24. Yanof, Z. A.: Blood Pyruvic Acid in Heart Disease, Arch. Int. Med. 69: 1005, 1942.
25. Turnock, D. M., and Welbourn, R. B.: The Effects of Glucose on Blood Pyruvic Acid Levels in Mild Thiamine Deficiency, J. Lab. & Clin. Med. 42: 261, 1953.
26. Friedemann, T. E., Ivy, A. C., Kinney, V. M., Sheft, B. B., and Harris, S. C.: Work at High Altitude. VI. Quart. Bull., Northwestern Univ. M. School 23: 448, 1949.
27. Williams, R. H., Egana, E., Robinson, P., Asper, S. P., and Dutoit, C. H.: Alterations in Biological Oxidations in Thyrotoxicosis, J. Clin. Invest. 21: 623, 1942.
28. Parida, R. K.: Unpublished experimental data.
29. Amatuzio, D. S., and Nesbitt, S.: Study of Pyruvic Acid in Blood and Spinal Fluid of Patients With Liver Disease, With and Without Hepatic Coma, J. Clin. Invest. 29: 1486, 1950.
30. Amatuzio, D. S., Shrifter, N., Stutzman, F. L., and Nesbitt, S.: Blood Pyruvic Acid Response to Intravenous Glucose or Insulin in the Normal and in Patients With Liver Disease and With Diabetes Mellitus, J. Clin. Invest. 31: 751, 1952.
31. Steigman, F., Popper, H., Hernandez, R., and Schulman, B.: Flocculation Tests in the Diagnosis of Hepatobiliary Disease, Gastroenterology 13: 9, 1949.

HEMOGLOBIN H

Clinical, Laboratory, and Genetic Studies of a Family With a Previously Undescribed Hemoglobin

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A NEW hemoglobinopathy has been observed in three members of a Chinese family. The clinical findings, genetic pattern, and characteristics of the abnormal hemoglobin are presented here.

Since 1949, when Pauling and associates[1] characterized sickle-cell hemoglobin, additional abnormal hemoglobins C,[2] D,[3] E,[4, 5] and G[6] have been described. At pH 8.6 these five all have an electrophoretic mobility of less than that of the normal adult type, or A hemoglobin. Sickle-cell hemoglobin also differs from normal adult hemoglobin and from all others previously described in that it has a markedly reduced solubility under conditions of reduced oxygen tension.[7] The abnormal hemoglobin[8] observed in our patients differs from hemoglobins S, C, D, E, and G in having a more rapid electrophoretic mobility at pH 8.6 than normal adult hemoglobin. It resembles sickle-cell hemoglobin in that it is less soluble in the reduced form.

METHODS

Red cell counts, hemoglobins, hematocrits, and reticulocyte counts were determined by standard methods.[9] Quantitative osmotic fragilities were made by the method of Hunter.[10] Sickle-cell preparations were made by mixing equal volumes of oxalated blood and 2 per cent sodium metabisulfite. Red cell survival was determined by reinjecting the patient's cells labeled with Cr^{51} according to a slight modification of the method of Sterling and Gray.[11] Fetal hemoglobin was measured by the alkaline denaturation method of Singer and associates.[12] The solubility test for hemoglobin S developed by Itano[13] was applied. Electrophoretic analyses were performed on hemolysates prepared by the method of Drabkin,[14] as modified by Havinga[15]; these were clarified by centrifugation at 12,000 g. for fifteen to twenty minutes and converted to carbonmonoxyhemoglobin by equilibrating the solutions in an atmosphere of 100 per cent carbon monoxide. The Klett-Tiselius apparatus was used with the 11 ml. cell. Pure carbonmonoxyhemoglobin fractions obtained from the electrophoretic cell by a previously described sampling device[16] were used for absorption spectrum analyses in the Beckman Model DU spectrophotometer and for ultracentrifugal analysis with the Spinco analytical ultracentrifuge.* Paper electrophoretic studies were made with the Spinco, Model R, Durrum type apparatus on clarified hemolysates without conversion to carbonmonoxyhemoglobin.

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TABLE I. HEMATOLOGICAL VALUES OF MEMBERS OF FAMILY WITH HEMOGLOBIN H

FAMILY NO.*	RED BLOOD CELLS	HEMOGLOBIN (GM./100 ML.)	HEMATOCRIT (ML./100 C.C.)	COLOR INDEX	VOLUME INDEX	RETICULOCYTES PER CENT	MICROCYTES AND TARGET CELLS	HEMOGLOBIN H PER CENT OF TOTAL	FETAL HEMOGLOBIN PER CENT OF TOTAL	BLOOD TYPES		
										ABO	MN	rH
10	5.0	8.0	30.3	0.56	0.70	22.1	++++	38.0	2.1	A	M	DCE
9	6.4	11.0	-	0.59	-	18.3	++++	35.0	2.6	A	M	DCE
7	5.4	11.3	-	0.71	-	13.9	++++	40.0	3.86	A	M	DCe
1	4.6	12.0	-	0.91	-	1.5	-	0.0	1.6	A	M	DCE
2	5.8	12.4	-	0.73	-	0.5	-	0.0	1.5	A	M	DCE
8	6.4	15.0	-	0.80	-	1.9	-	0.0	1.4	A	M	DCe
11	5.2	13.1	40.9	0.86	0.96	0.9	-	0.0	-	A	MN	DCE
12	5.8	10.1	34.0	0.72	0.81	3.9	++	0.0	0.0	A	MN	DCE

*Family numbers correspond to those in Fig. 1.

CASE HISTORIES

Fig. 1 is the genetic diagram of the family studied. All members were full-blooded Chinese, whose ancestors came from Canton. There was no history of consanguineous marriages.

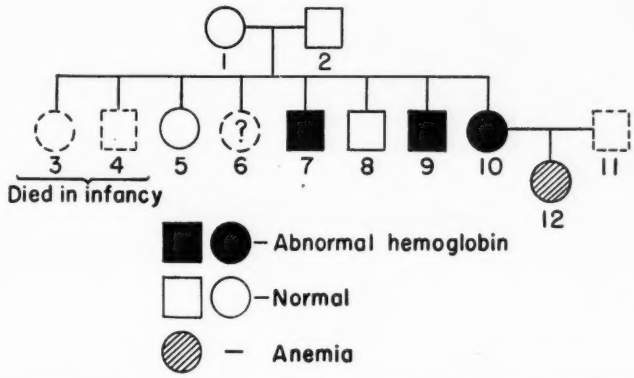

Fig. 1.—Genetic diagram of family with hemoglobin H. Subject 10 is propositus.

The propositus (No. 10), a 30-year-old housewife, was first seen on March 23, 1954. Pertinent history included lifelong easy fatigability, episodic scleral icterus, and known anemia which had not responded to several courses of oral and parenteral hematinics including iron and liver extract. Except for small stature (height 60 inches, weight 90 pounds) and an enlarged spleen, which extended 5 cm. below the left costal margin, physical findings were normal. The results of laboratory tests are listed in Table I. The significant findings were a hypochromic microcytic anemia with extreme poikilocytosis, microcytosis, and numerous target cells, as illustrated in Fig. 2, *A*. Fetal hemoglobin was 2.1 per cent and electrophoretic analysis demonstrated 38 per cent of the hemoglobin to be abnormal. Sickling preparation showed no sickle cells. Van den Bergh's test showed total bilirubin of 0.6 mg. with 0.4 mg. indirect reacting bilirubin. Marrow was cellular; there was moderate erythroid hyperplasia and increased amounts of hemosiderin.

The propositus' husband (No. 11) was a 34-year-old man who was well. All laboratory studies on his blood were normal.

The propositus' 8-year-old daughter (No. 12) had been well except for minor childhood illnesses. She had no pallor, icterus, splenomegaly, or hepatomegaly but did have a mild hypochromic anemia (Table I) which had not responded to a therapeutic trial of oral iron. The red cells (Fig. 2, *B*) had less hypochromia and anisocytosis than were present in the mother's cells. The blood contained no demonstrable abnormal hemoglobin.

Osmotic fragility curves of Subjects 10, 11, and 12 are plotted in Fig. 3; that of the propositus and her daughter are similar in that there was increased red cell resistance to hypotonic saline, while that of the propositus' husband was normal.

Subject 9, the 25-year-old brother of the propositus, had been known to have anemia and splenomegaly since 1952. In February, 1954, he was hospitalized because of anemia and bronchopneumonia. Blood counts were 1.63 million erythrocytes; 3.5 Gm. of hemoglobin; 34,000 leukocytes, predominately neutrophils; 14 nucleated erythrocytes per 100 leukocytes; and marked hypochromia with anisocytosis. He was given antibiotics and a total of eleven blood transfusions, and he recovered. Blood samples for our studies were obtained two months later, in May, 1954. These showed reticulocytosis, hypochromia, and morphologic red cell changes similar to those of the propositus, 2.6 per cent fetal hemoglobin and 35 per cent abnormal hemoglobin.

Subject 8, the 40-year-old brother of the propositus, had always been well. There were no detectable abnormalities of the blood.

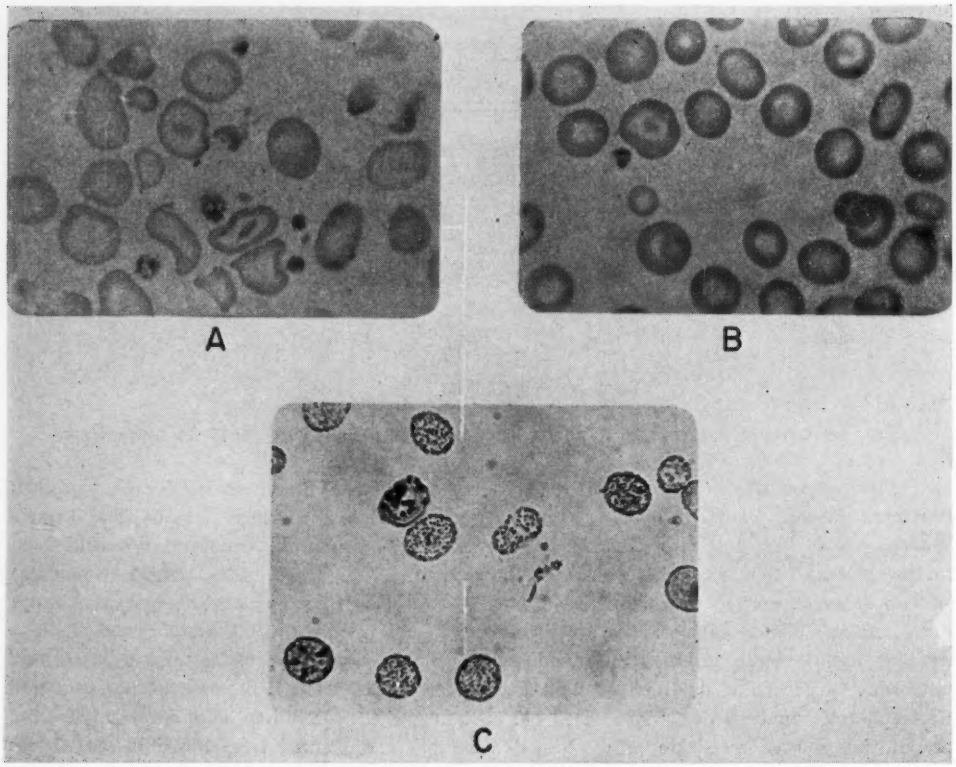

Fig. 2.—*A*, Wright-stained smear of blood from propositus (×525); *B*, Wright-stained smear of blood from Subject 12 (×525); *C*, reticulocyte preparation of blood from propositus (×500).

Subject 7, the 24-year-old brother of the propositus, had been known to be anemic since 1947. Like the propositus and Subject 9, he was of small stature with a height of 56 inches, and a weight of 112½ pounds. He had an enlarged spleen which extended 4 cm. below the left costal margin, and he had similar red cell changes on the stained blood smear. He had 3.9 per cent fetal hemoglobin and 40 per cent of the hemoglobin was abnormal by electrophoresis. Osmotic fragility of the red cells was decreased. Red cell survival study with Cr^{51}-labeled cells was markedly shortened, as shown in Fig. 4.

We have not had the opportunity to study Subjects 5 and 6 except that a hemoglobin solution from Subject 5 was shown to be normal by paper electrophoresis. Subject 6, the 35-year-old sister of the propositus, is said to have an "allergy" and to be receiving iron and liver shots. Two siblings of the propositus (Subjects 3 and 4) died in infancy of unknown causes. The propositus' parents (Subjects 1 and 2) are both well and have no anemia. Free electrophoresis of carbonmonoxyhemoglobin from subjects 1 and 2 shows no abnormal hemoglobin; electrophoretic patterns of hemolysates from the propositus' parents (1, 2) and daughter (12) are shown in Fig. 5. All three show a small component moving with an electrophoretic mobility less than that of hemoglobin A. This component is also found in hemolysates from normal blood. Although not shown in Fig. 6, *a*, it was later observed in hemolysates from Subjects 7, 9, and 10. This component may be identical to the slow

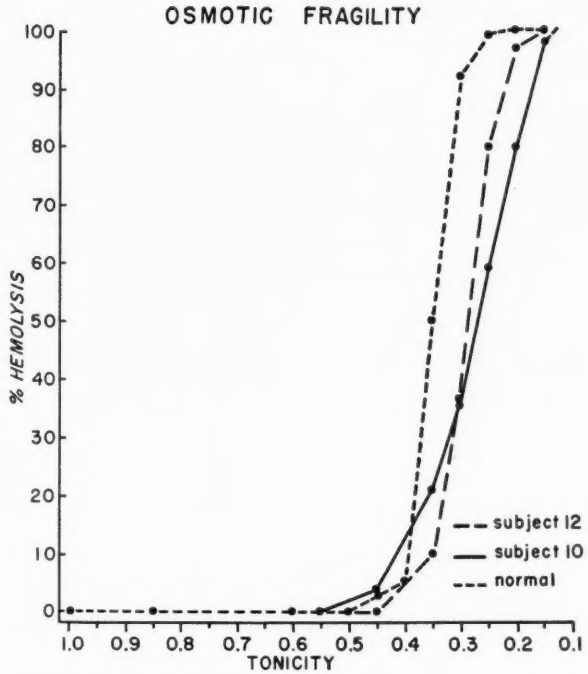

Fig. 3.—Osmotic fragility in saline of erythrocytes from Subjects 10 and 12.

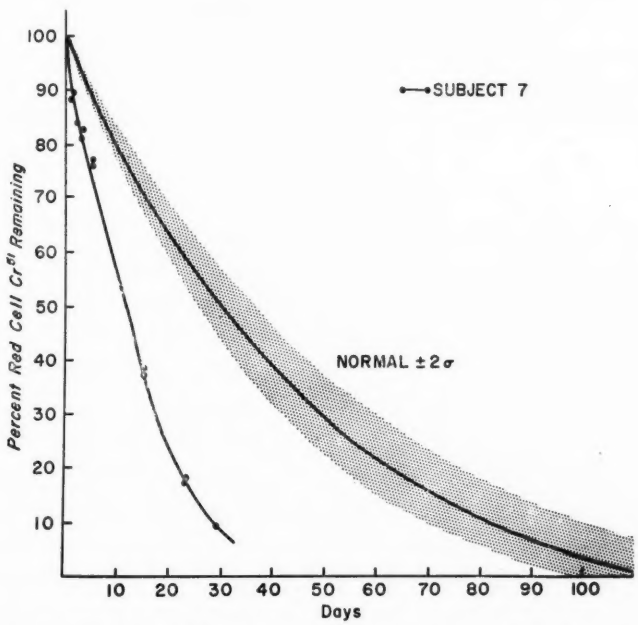

Fig. 4.—Survival of Cr^{51}-labeled red cells from Subject 7 in his own circulation.

component described by Kunkel and Wallenius[38] in normal adult blood. Pertinent laboratory data for all subjects studied are tabulated in Table I. Joint, abdominal, or back pain has not occurred in the three members who have the abnormal hemoglobin.

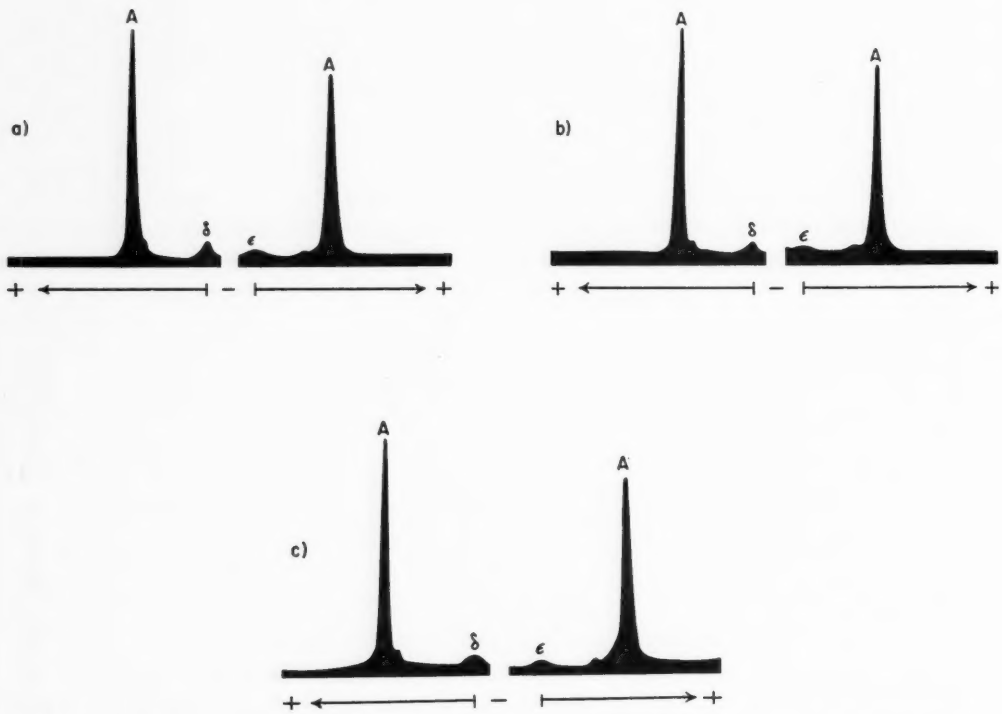

Fig. 5.—Electrophoretic patterns of carbonmonoxyhemoglobin made in Klett-Tiselius 11 ml. cell at 1° C. with potential gradient of 7.5 volts per sq. cm. All were run in barbital buffer,[18] pH 8.6, ionic strength 0.1. *a*, Subject 1, 150 min.; *b*, Subject 2, 150 min.; *c*, Subject 12, 180 min.

SPECIAL STUDIES

Electrophoretic Behavior.—The striking resemblance of Wright's stained blood films from Patients 7, 9, and 10 to hereditary leptocytosis trait and the relatively severe clinical symptoms of these patients suggested the possibility of a combination of an abnormal hemoglobin with hereditary leptocytosis trait. Electrophoretic analyses were therefore undertaken to investigate this possibility. An analysis performed on the carbonmonoxyhemoglobin of Patient 10 with the Klett-Tiselius apparatus in barbital buffer of pH 8.6 and ionic strength 0.1[18] revealed the existence of two hemoglobins (Fig. 6, *a*). Identical patterns under the same experimental conditions were obtained from Patients 7 and 9. The slower peak is normal adult hemoglobin A. It migrates with added normal hemoglobin[8] and its electrophoretic mobility curve *(vide infra)* is identical with that given by Pauling and associates[1] for normal adult carbonmonoxyhemoglobin. No abnormal hemoglobin was demonstrated under the same experimental conditions in Subjects 1, 2, 8, and 12. The more rapidly migrating hemoglobin is

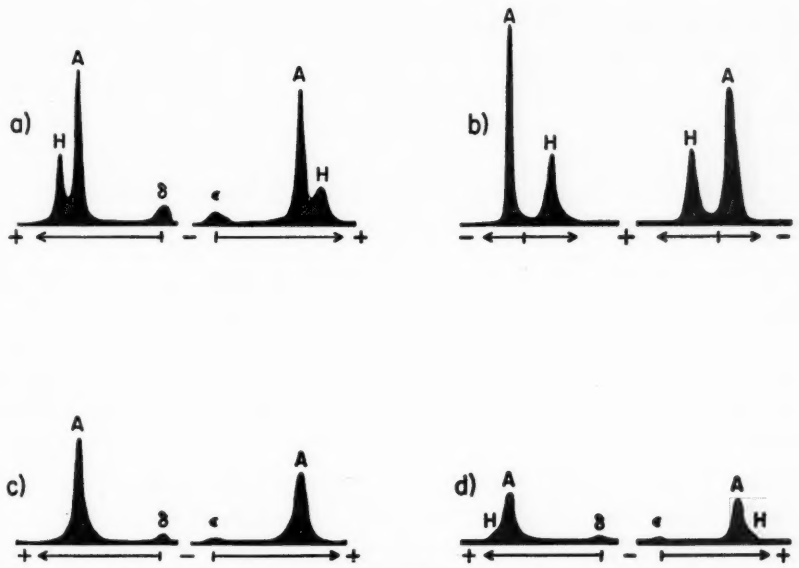

Fig. 6.—*a-d*, Electrophoretic patterns of carbonmonoxyhemoglobin from Subject 10 made in Klett-Tiselius 11 ml. cell at 1° C. with potential gradient of 7.5 volts per sq. cm. *a*, Barbital buffer,[18] pH 8.6, ionic strength 0.1, 270 min., *b*, phosphate buffer,[24] pH 6.0, ionic strength 0.1, 270 min.; *c*, same conditions as in Fig. 5, *a* except that carbonmonoxyhemoglobin has been stored five months at 4° C.; *d*, Same conditions as in Fig. 6, *a* except that carbonmonoxyhemoglobin has been subjected to solubility test for hemoglobin S.[13]

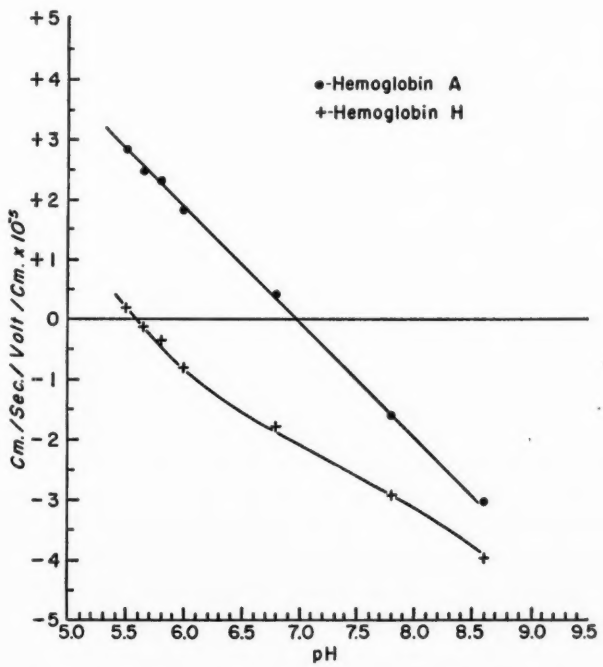

Fig. 7.—Electrophoretic mobilities of pure A and pure H carbonmonoxyhemoglobin. All runs were made in Klett-Tiselius 11 ml. cell at 1° C. with potential gradient of 7.5 volts per sq. cm. Buffers for pH 5.0 to 8.0 are those of Miller[24] and for pH 8.6, barbital buffer.[18] All have ionic strength of 0.1.

abnormal and we have designated it "hemoglobin H."* The amount of hemoglobin H in hemolysates from Patients 7, 9, and 10 is 35 per cent to 40 per cent of the total hemoglobin, as noted in Table I. Since different proteins may have the same electrophoretic mobility at a certain pH, it is of paramount importance that every new hemoglobin be investigated over a wide pH range and be characterized by its isoelectric point and its electrophoretic mobility curve. For this reason electrophoretic analyses of the carbonmonoxyhemoglobins of Patient 10 were performed with the Klett-Tiselius apparatus throughout the pH range from 5.5 to 8.6. The buffer systems of ionic strength 0.1 described by Miller and Golder[24] were utilized except for pH 8.6 where the previously mentioned barbital buffer was used. The analyses were carried out at a temperature of 1° C. and the hemoglobins were allowed to migrate for four and one-half to seven hours under the influence of a potential gradient of 7.5 volts per square centimeter. The pattern obtained at pH 6.0 is shown in Fig. 6, *b*. The mean electrophoretic mobility was calculated from the ascending and the descending limbs for both the normal hemoglobin A and the abnormal H. The curves obtained by plotting the electrophoretic mobility of each hemoglobin against pH are shown in Fig. 7. The two curves are widely separated from each other and the difference between their isoelectric points is large. The isoelectric point and the mobility curve of the normal hemoglobin A are not significantly different from those reported by Pauling.[1] The isoelectric point of hemoglobin H is in the vicinity of pH 5.60 (between pH 5.5 and 5.65), which is markedly lower than that of hemoglobin A.

Absorption Spectra.—The absorption spectrum of hemoglobin H (obtained in pure form from the electrophoretic cell with the sampling device[16]) was compared with that of normal adult hemoglobin from 660 to 220 mμ with the Beckman DU spectrophotometer. There is no significant difference between the two spectra; both have the same maxima and minima.

Ultracentrifugal Analyses.—Pure fractions of hemoglobin A and hemoglobin H obtained from the electrophoretic cell were analyzed simultaneously with a Spinco analytic ultracentrifuge.[17] It was observed that hemoglobin H has a slightly higher sedimentation constant than hemoglobin A. Whether this is due to a higher molecular weight, different molecular shape, or to an early phase of denaturation and aggregation of hemoglobin H is not known. Further studies are planned to investigate these possibilities.

Stability and Solubility.—When clarified hemolysate from Patients 7, 9, and 10 was allowed to stand in the refrigerator (4° C.) or at room temperature, a brown precipitate gradually formed. Electrophoretic analyses of the supernatant solution, after removal of the precipitate by centrifugation, revealed that the amount of hemoglobin H was progressively diminishing, and that it finally disappeared completely. This is shown in Fig. 6, *c*, which is the same sample of hemoglobin shown in Fig. 6, *a*, reanalyzed under identical experimental condi-

*Other abnormal hemoglobins, which like hemoglobin H migrate faster than normal adult hemoglobin at pH 8.6, have been recently observed by Page, Rucknagel, and Jensen,[19] Motulsky,[20] Sturgeon and Bergren,[21] Thorup,[22] and Fessas and Gouttas.[23] Of these hemoglobins, those of Jensen and associates, and Thorup have different electrophoretic mobilities than that of hemoglobin H, either at pH 8.6, pH 7.0, or at both pH's, on paper electrophoresis.

tions after five months' storage at 4° C. Acidhematin analyses performed on the precipitate revealed that it is precipitated hemoglobin. When pure hemoglobin H and hemoglobin A were obtained from the electrophoretic cell, only hemoglobin H precipitated rapidly on storage. Attempts to bring the precipitated hemoglobin back into solution have so far been unsuccessful. Thus, it appears that hemoglobin H is unstable and in time precipitates in an irreversibly denatured form. The denaturation proceeds faster on the acid side of the pH scale. Practically complete denaturation of hemoglobin H occurs by a single freezing and thawing. All attempts to prevent this denaturation by storing the hemoglobin in buffers of different ionic make-up, pH, or ionic strength have failed.

The solubility test for hemoglobin S,[13] performed with hemolysates containing hemoglobin H, gave a flocculent precipitate in the 2.24 M phosphate, which after centrifugation at high speed separated as a brown layer at the surface. Attempts to redissolve the precipitate by suspending it in isotonic saline and oxygenation were unsuccessful. The clarified hemoglobin solution, after the removal of the precipitate, was dialyzed against cold water to remove the buffer salts and then against barbital buffer of pH 8.6, ionic strength 0.1. An electrophoretic analysis performed on this solution disclosed that the bulk of hemoglobin H had been precipitated (Fig. 6, *d*), leaving only a very small amount in solution.

Inclusion Bodies.—Reticulocyte and sickling preparations of the subjects who have hemoglobin H were observed to have intraerythrocytic inclusion bodies in nearly all red cells, as can be seen in Fig. 2, *C*. Wright's stained smears did not exhibit these bodies, and at first they were discounted as being due to crenation. This misconception was enforced by another observation. Cells from oxalated venous blood, when freshly mixed with reticulocyte stain or sodium metabisulfite, resembled the cells shown on Wright's stained smears (Fig. 2, *A*). A period of fifteen to thirty minutes was required before the intraerythrocytic bodies appeared. Because of the solubility and stability characteristics of the purified abnormal hemoglobin, an attempt was made to determine whether the factors leading to formation of these bodies are the same as those which cause denaturation and precipitation of hemoglobin H. Wet mounts of freshly drawn red cells from the propositus suspended in reticulocyte stain,[9] in 2 per cent sodium metabisulfite, and in the patient's plasma were observed by phase microscopy. In all three types of preparation, beginning in ten to thirty minutes after the mount was set up, a coalescence of intraerythrocytic material into many refractile globules which oriented themselves at the periphery of the cell was observed. No crystalline structure was discernible; the bodies were seen to take up a blue color in the reticulocyte preparation. When clarified oxyhemoglobin solutions from the propositus were mixed with similar solutions of reticulocyte stain or 2 per cent sodium metabisulfite, a precipitate formed. As in the precipitates formed in the solubility test, these could not be redissolved. Conversion of the washed precipitate to acidhematin, and quantification as hemoglobin accounted for 32.7 per cent of the

original hemoglobin concentration. Pure hemoglobin H obtained from the electrophoretic cell was precipitated by these agents, while pure hemoglobin A was not.

If the formation of such inclusion bodies, which appear to represent denatured hemoglobin H, is of any clinical significance, they should occur in vivo. To test this possibility, capillary blood from Subjects 7 and 10 was observed in a petrolatum-rimmed cover slip preparation as soon as possible (thirty to sixty seconds) after finger-stick. This was done before and after application of a tourniquet for three minutes. In both subjects, 60 to 80 per cent of the red cells contained the inclusion bodies when such preparations were first observed, and over a period of ten minutes all the red cells were affected. No significant difference was noted in the number of cells containing inclusions in capillary blood before or after tourniquet applications.

Red Cell Survival.—Red cells from Subject 7 were labeled with radioactive chromium, washed three times in physiologic saline solution, and reinjected. Blood volume and red cell mass were calculated from the activity of a blood sample obtained after fifteen minutes and were 4,250 ml. (83.5 ml. per kilogram) and 1,615 ml. (31.7 ml. per kilogram), respectively. Fig 4 is the survival curve of these cells and the normal range we have observed using this method.[25]

Osmotic Fragility.—Because of the morphologic similarity of red cells from Subjects 7, 9, 10, and 12 to those of hereditary leptocytosis, quantitative osmotic red cell fragilities were made on Subjects 7, 10, 11, and 12. All except Subject 11, who is normal by all other criteria, show a definite increased resistance to hypotonic saline solution. This is illustrated in Fig. 3.

GENETICS

One or more pairs of somatic genes appear to control hemoglobin synthesis. Neel's[26] hypothesis of the occurrence of the gene for sickling in the heterozygous or homozygous form for sicklemia and sickle-cell anemia, respectively, fits both the mode of transmission and the quantities of abnormal hemoglobin found in the two conditions. Similar genetic patterns have been observed for hemoglobin C,[2] D[3], E,[4] and G.[5] The observation that sickle-cell trait patients usually have 40 per cent, rather than the theoretical 50 per cent, abnormal hemoglobin and may have as little as 5 per cent sickle-cell hemoglobin implies that the effectiveness of the abnormal gene may be less than that of the normal. Rich[27] has suggested that the presence of the gene for hereditary leptocytosis trait, which appears to follow similar genetic patterns, interferes with the synthesis of normal hemoglobin. In a few cases of simultaneous occurrence of hereditary leptocytosis trait and the trait for hemoglobin S[28-32] or hemoglobin C,[33] the quantity of the abnormal hemoglobin has been greater than the theoretical 50 per cent.

The pattern of inheritance observed in the family reported here is not easily explainable on these mechanisms. Absence of the abnormal hemoglobin

and of any significant anemia in Subjects 1 and 2 could be accounted for by several hypotheses and our data are not adequate to decide among these:

1. Hemoglobin H is transmitted as a recessive characteristic and in the homozygous form causes production of only 30 to 40 per cent abnormal hemoglobin.

2. Hemoglobin H is transmitted like the other abnormal hemoglobins but was not observed in either parent because of: (a) False paternity. This was not substantiated by history, and the ABO, MN, and Rh blood types do not exclude paternity or sibship. (b) Occurrence of the hemoglobin H gene as a mutation in the germ-cell layer of Subjects 1 or 2 without involvement of their somatic cells. (c) Marked variation of penetrance of the abnormal hemoglobin so that an insufficient amount is synthesized by Subjects 1 or 2 to be demonstrable by electrophoresis. (d) Penetrance of the abnormal hemoglobin is increased by the presence of the gene for hereditary leptocytosis. The morphologic changes and the osmotic fragility curves of the propositus, her two brothers (7 and 9), and her daughter would be compatible with this genetic make-up. Her father (2) has definite hypochromia but no anemia and no morphologic changes of the red cells on stained blood smears. He may have leptocytosis trait, which is transmitted to his children (7, 9, and 10) and to his granddaughter (12). This interpretation would imply that the propositus' mother (1) carries the genetic abnormality which causes no demonstrable abnormality in her hemoglobin, but when present together with the gene for leptocytosis trait in her children (7, 9, and 10) produces the clinical picture described.

Until more family studies are completed, the mode of inheritance of hemoglobin H remains uncertain. At present the most likely possibility is the simultaneous occurrence of H trait with hereditary leptocytosis trait in Subjects 7, 9, and 10.

DISCUSSION

Morphologic alterations of the red cells have been described in hemoglobinopathies due to the presence of hemoglobins S and C. The pathogenesis of the occlusive vascular complications and the shortened red-cell survival has been attributed to the sickling phenomenon in sickle-cell anemia, and this in turn is thought to be due to the tactoid formation of S hemoglobin in the reduced form.[34] Intraerythrocytic crystals[35] have been observed in homozygous C disease following splenectomy. Whether the occurrence of these bodies is related to the hemolytic process demonstrated in this condition[36] is not apparent. Minnich and associates[37] described intraerythrocytic inclusion bodies in a group of patients studied in Thailand, some of whom were later shown to have a combination of hereditary leptocytosis trait and hemoglobin E.[4] Again the relationship of the bodies to the hemolytic process is unknown.

The appearance of intraerythrocytic inclusion bodies in our patients occurs under conditions which cause irreversible denaturation of hemoglobin H. The fact that such red-cell changes are present in freshly drawn capillary blood suggests that hemoglobin H exerts an in vivo effect and may cause the observed shortened red-cell survival. We have no direct proof that the cells containing

inclusion bodies of precipitated hemoglobin H are more susceptible to hemolysis, but it is presumed that they are. Whether the formation of the bodies represents an irreversible change is also not proved. They are not seen in samples of venous blood which have been shaken with an anticoagulant, probably because the blood is oxygenated in this process. Since the inclusion bodies are present in capillary blood it seems probable that the formation of intracellular aggregates of hemoglobin H is reversible. More studies are needed to correlate the in vitro changes with factors such as oxygen tension and pH which may alter the cells in the circulation.

It is possible to rephrase in purely physicochemical terms this concept of the pathogenesis of hemoglobin H disease. Instability of hemoglobin H has been demonstrated; pH and oxygen tension alter the rate at which the molecule denatures, but irreversible precipitation occurs in spite of attempts to control these factors. It is conceivable that hemoglobin H molecules, as synthesized, have unstable surface forces or chemical bonds which may break at random because of environmental factors (especially at sites in vivo where lowered pH and reduced oxygen tension occur), resulting in an alteration of the original molecular form. This would lead to a definite half-life of hemoglobin H analagous to that of a radioactive isotope. The denatured form, being less soluble, may precipitate to form the observed intraerythrocytic inclusion bodies when a critical concentration is reached. Subsequent destruction of the cells containing such bodies may account for the shortened survival time of the hemoglobin H containing erythrocytes.

SUMMARY

Eight members of a Chinese family have been studied. Three of these have a previously undescribed hemoglobinopathy characterized by hypochromic microcytic anemia refractory to iron therapy, reticulocytosis, splenomegaly, increased red-cell resistance to hypotonic saline solution, and shortened red-cell survival. The inheritance of this condition is not typical of a dominant gene. The abnormal hemoglobin H accounts for 35 to 40 per cent of the total; fetal hemoglobin is present in slightly increased amounts, and the remainder is normal adult hemoglobin. Hemoglobin H differs from normal adult hemoglobin in having a more rapid electrophoretic mobility on the alkaline side of the pH range, and a lower isoelectric point.

Hemoglobin H precipitates in an irreversible form from solution and in the red cells, forming intraerythrocytic inclusion bodies. This alteration occurs at a more rapid rate with increasing acidity or decreasing oxygen tension but appears to be due to an inherent molecular instability. Attempts are made to relate the characteristics of the abnormal hemoglobin to the morphologic changes in the red cells and to the pathogenesis of the disease.

ADDENDUM

Since preparation of this manuscript, we have learned of independent observations by Fessas[23] and Goutas of a Greek family, some members of which have leptocytosis trait and some members have an abnormal hemoglobin. The latter have a clinical picture and

laboratory findings very similar to the patients described here. Electrophoretic mobility and instability of the abnormal hemoglobin, as shown by paper electrophoresis, appear to be similar if not identical with our findings. They also observed in vitro formation of erythrocyte inclusion bodies in brilliant cresyl blue preparations.

We are indebted to Drs. F. M. Bryant, Marian Hayes, W. H. Perry, and D. B. Roxburgh for their help in obtaining specimens for these studies, and to Dr. Arthur Frisch for doing blood-typing studies. We wish to acknowledge the work done by Miss Marie Duerst, Mrs. Charlotte Moffat, Miss Barbara Hoffman, John Brooke, Miss Sophia Langas, and other members of our technical staff who helped carry out the special studies described.

REFERENCES

1. Pauling, L., Itano, J. A., Singer, S. J., and Wells, I. C.: Sickle Cell Anemia, a Molecular Disease, Science **110**: 543, 1949.
2. Itano, H. A., and Neel, J. V.: New Inherited Abnormality of Human Hemoglobin, Proc. Nat. Acad. Sc. **36**: 613, 1950.
3. Itano, H. A.: A Third Abnormal Hemoglobin Associated With Hereditary Hemolytic Anemia, Proc. Nat. Acad. Sc. **37**: 775, 1951.
4. Chernoff, A. I., and Minnich, V.: Hemoglobin E, a Hereditary Abnormality of Human Hemoglobin, Science **120**: 605, 1954.
5. Itano, H. A., Bergren, W. R., and Sturgeon, P.: Identification of a Fourth Abnormal Hemoglobin, J. Am. Chem. Soc. **76**: 2278, 1954.
6. Edington, G. M., and Lehmann, H.: Haemoglobin G.: A New Haemoglobin Found in a West African, Lancet **267**: 173, 1954.
7. Perutz, M. F., and Mitcheson, J. M.: State of Haemoglobin in Sickle Cell Anemia, Nature **166**: 677, 1950.
8. Rigas, D. A., Koler, R. D., and Osgood, E. E.: A New Hemoglobin Possessing a Higher Electrophoretic Mobility Than Normal Adult Hemoglobin, Science **121**: 372, 1955.
9. Osgood, E. E.: Laboratory Diagnosis, Philadelphia, 1940, The Blakiston Company.
10. Hunter, F. T.: Photoelectric Method for Quantitative Determination of Erythrocyte Fragility, J. Clin. Invest. **19**: 691, 1940.
11. Sterling, K., and Gray, S. J.: Determination of the Circulating Red Cell Volume in Man by Radioactive Chromium, J. Clin. Invest. **29**: 1614, 1950.
12. Singer, K., Chernoff, A. I., and Singer, L.: Studies on Abnormal Hemoglobins. I. Their Demonstration in Sickle Cell Anemia and Other Hematologic Disorders by Means of Alkali Denaturation, Blood **6**: 413, 1951.
13. Itano, H. A.: Solubilities of Naturally Occurring Mixtures of Human Hemoglobin, Arch. Biochem. **47**: 148, 1953.
14. Drabkin, D. L.: Spectrophotometric Studies. XIV. Crystallographic and Optical Properties of the Hemoglobin of Man in Comparison With Those of Other Species, J. Biol. Chem. **164**: 703, 1946.
15. Havinga, E.: Comparison of the Phosphorus Content, Optical Rotation, Separation of Hemes and Globin, and Terminal Amino Acid Residues of Normal Adult Human Hemoglobin and Sickle Cell Anemia Hemoglobin, Proc. Nat. Acad. Sc. **39**: 59, 1953.
16. Rigas, D. A., Maupin, T. J., and Heller, Carl, G.: A Mechanical Sampling Device for Use With the Standard Electrophoresis Apparatus, J. LAB. & CLIN. MED. **39**: 492, 1952.
17. deLalla, Oliver F.: Personal communication.
18. Longsworth, L. G.: Recent Advances in the Study of Proteins by Electrophoresis, Chem. Rev. **30**: 323, 1942.
19. Page, E. B., Rucknagel, D. L., and Jensen, W. N.: Clinical and Genetic Studies of a New Inherited Abnormality of Hemoglobin Characterized by a Rapid Electrophoretic Mobility, Clin. Res. Proc. **3**: 67, 1955.
20. Motulsky, A.: Personal communication, 1954.
21. Sturgeon, P., and Bergren, W. R.: Personal communication, 1955.
22. Thorup, O. A.: Personal communication, 1955.
23. Fessas, P., and Gouttas, A.: Personal communication, 1955.
24. Miller, G. L., and Golder, R. H.: Buffers of pH 2 to 12 for Use in Electrophoresis, Arch. Biochem. **29**: 420, 1950.
25. Kurtz, M.: Unpublished observations.
26. Neel, J. V.: The Inheritance of Sickle Cell Anemia, Science **110**: 64, 1949.
27. Rich, A.: Studies on the Hemoglobin of Cooley's Anemia and Cooley's Trait, Proc. Nat. Acad. Sc. **38**: 187, 1952.
28. Silvestroni, E., and Bianco, I.: Genetic Aspects of Sickle Cell Anemia and Microdrepanocytic Disease, Blood **7**: 429, 1952.

29. Powell, W. N., Rodarte, J. G., and Neel, J. V.: The Occurrence in a Family of Sicilian Ancestry of the Traits for Both Sickling and Thalassemia, Blood **5**: 887, 1950.
30. Sturgeon, P., Itano, H. A., and Valentine, W. N.: Chronic Hemolytic Anemia Associated With Thalassemia and Sickle Traits, Blood **7**: 350, 1952.
31. Neel, J. V., Itano, H. A., and Lawrence, J. S.: Two Cases of Sickle Cell Disease Presumably Due to the Combination of the Genes for Thalassemia and Sickle Cell Hemoglobin, Blood **8**: 434, 1953.
32. White, J. C., and Beaven, G. H.: A Review of the Varieties of Human Haemoglobin in Health and Disease, J. Clin. Path. **7**: 175, 1954.
33. Singer, K., Kraus, A. P., Singer, L., Rubinstein, H. M., and Gohlberg, S. R.: Studies on Abnormal Hemoglobins. X. A New Syndrome: Hemoglobin C-Thalassemia Disease, Blood **9**: 1032, 1954.
34. Harris, J. W.: Studies on Destruction of Red Blood Cells. VIII. Molecular Orientation in Sickle Cell Hemoglobin Solutions, Proc. Soc. Exper. Biol. & Med. **75**: 197, 1950.
35. Diggs, L. W., Kraus, A. P., Morrison, D. B., and Rudnicki, R. P. T.: Intraerythrocytic Crystals in a White Patient With Hemoglobin C in the Absence of Other Types of Hemoglobin, Blood **9**: 1172, 1954.
36. Terry, D. W., Motulsky, A. G., and Rath, C. E.: Homozygous Hemoglobin C. A New Hereditary Hemolytic Disease, New England J. Med. **251**: 365, 1954.
37. Minnich, V., Na-Nakorn, S., Chongchareonsuk, S., and Kochaseni, S.: Mediterranean Anemia. A Study of Thirty-two Cases in Thailand, Blood **9**: 1, 1954.
38. Kunkel, H. G., and Wallenius, G.: New Hemoglobin in Normal Adult Blood, Science **122**: 288, 1955.

LABELING HUMAN ERYTHROCYTES WITH RADIOCHROMIUM*

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FOR estimation of bodily erythrocyte mass with Cr^{51}-labeled erythrocytes,[1] it is important that the amount of the labeling agent bound to the cells be known.[2] For the evaluation of erythrocyte survival[3, 4] or of alimentary-tract bleeding,[5-7] it is not important if a small amount of the Cr^{51} is unattached to the cells, for the noncellular Cr^{51} disappears almost completely from the circulating plasma of the patient within one to two days. The simplest way to label erythrocytes would be to add the radiochromium to whole blood. Such a technique was studied with the use of variations in anticoagulant, temperature, time, hematocrit value, and order of mixing the components. To study the rates of reaction, labeling was halted at will by the addition of ascorbic acid to the reaction mixtures.

METHODS

Inhibition of Labeling With Ascorbic Acid.—Varying amounts (0.1 to 20 mg.) of ascorbic acid in 1 ml. of 0.9 per cent NaCl were added to 3 ml. aliquots of whole blood (in citrate-dextrose anticoagulant), and this was followed by 1 ml. of $Na_2Cr^{51}O_4$ in NaCl solution.† In Fig. 1 it is shown that 8 or more milligrams of ascorbic acid per 3 ml. of blood was able to block Cr^{51}-labeling almost completely. More than 20 mg. of ascorbic acid per 3 ml. of blood caused hemolysis. The addition of 12 mg. of ascorbic acid per 3 ml. of blood was selected for routine analyses of inhibition.

Anticoagulants.—Two milliliter volumes of (a) sodium citrate (1.9 per cent in saline solution), (b) sodium salt of heparin (1 mg. in saline solution), (c) Baxter Laboratories, Inc. citrate-dextrose‡ (based on U.S.P. XIV), or (d) Strumia citrate-dextrose§ were added to 10 ml. of whole blood.

Temperature.—Water baths set at 26° and 37° C. were used.

Measurement of Cr^{51}-Labeling.—Following the addition of ascorbic acid to a mixture of whole blood and Cr^{51}, the specimen was centrifuged. The sedimented cells were washed three times with saline solution; then they were assayed for Cr^{51}. The Cr^{51} in the cells

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***Section of Clinical Pathology, Mayo Clinic and Mayo Foundation.

†Obtained from Abbott Laboratories under authorization of the Atomic Energy Commission.

‡Baxter Laboratories, Inc. solution:

Sodium citrate	1.37
Citric acid	.50
Dextrose	2.45
Water to make	100.

§Strumia solution[8]:

Disodium citrate, monohydrate	2.55
Citric acid	.80
Dextrose	1.20
Water to make	100.

was compared with the total amount of Cr^{51} added to the blood and was recorded as "Cr^{51} bound to cells (per cent)." Measurements of radioactivity were in a sodium iodide (thallium-activated) crystal of the well type. At our usual settings, the Cr^{51} yielded 1,400 counts per second (cps) per microcurie over a background of about 4 cps.

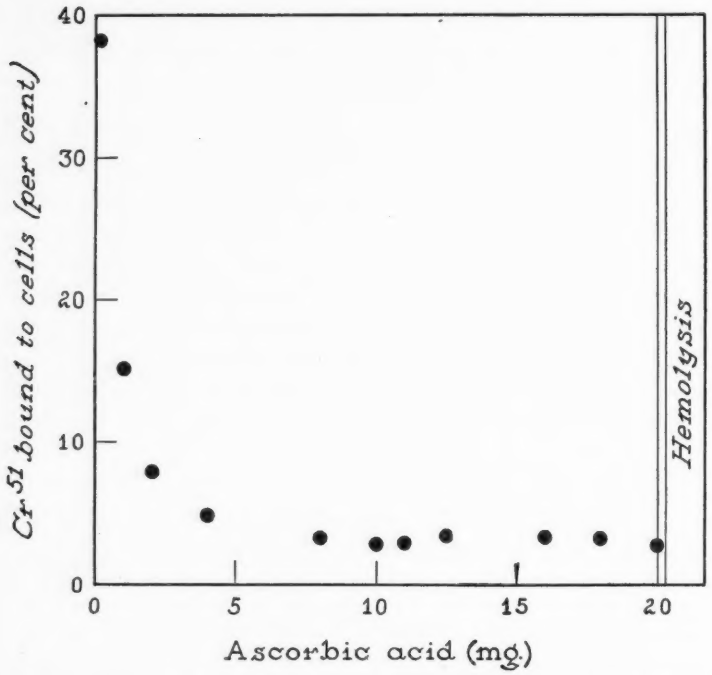

Fig. 1.—Inhibition of Cr^{51} labeling of erythrocytes with ascorbic acid. Variable amounts of ascorbic acid in solution were added to 3 ml. whole blood in citrate-dextrose solution; finally Cr^{51}, as chromate ion, was added. The percentage of maximal possible labeling was measured after fifteen minutes.

RESULTS

1. *Effect of Anticoagulants on Cr^{51}-Labeling.*—Use of the citrate-dextrose (ACD) anticoagulants led to a higher degree of Cr^{51}-labeling than when heparin or sodium citrate was used (Fig. 2). Acidification of heparin to the pH of the ACD solutions (5 to 5.5) did not significantly improve labeling of heparinized blood.

Fig. 3 illustrates the improved labeling of blood containing the Strumia type of ACD solution. In any ACD solution the erythrocytes swell, as determined by the hematocrit value; perhaps the slightly greater swelling which we found in association with the Strumia ACD solution accounts for its improved labeling.

2 and 3. *Effect of Temperature and Time on Cr^{51}-Labeling (Fig. 4).*—The erythrocytes of blood mixed with Strumia ACD solution were maximally labeled with Cr^{51} within about five minutes when the reaction occurred at 37° C. The peak was not reached until about fifteen minutes, at 26° C. However, the degree of labeling in fifteen minutes was practically the same at both temperatures. In all our studies with Strumia's ACD anticoagulant, maximal Cr^{51}-labeling seemed to occur within about fifteen minutes (Fig. 3).

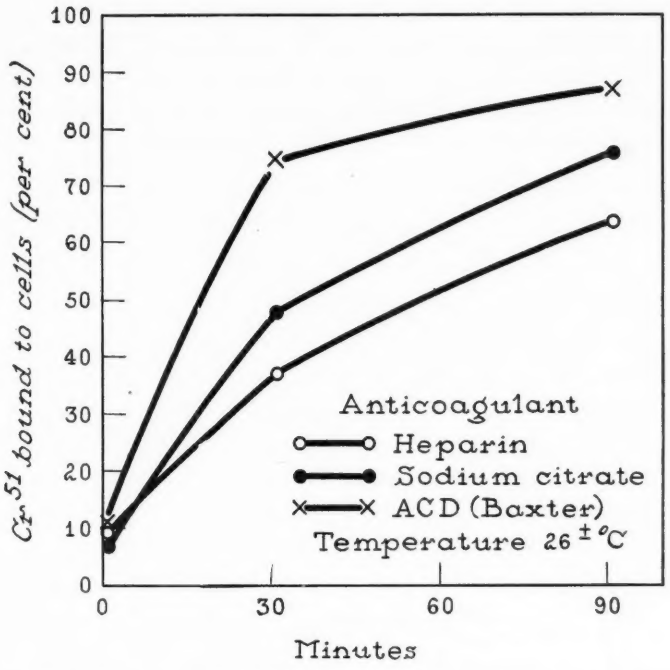

Fig. 2.—Comparison of various anticoagulants on the efficiency of labeling erythrocytes with Cr^{51}.

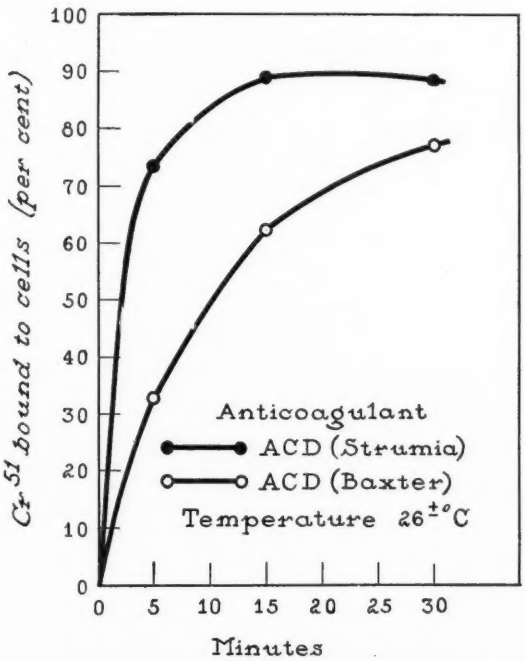

Fig. 3.—Comparison of citrate-dextrose anticoagulants of the Baxter and Strumia types on labeling of erythrocytes with Cr^{51}.

4. *Effect of Hematocrit and Cr^{51}-Labeling.*—In Fig. 5 is represented the maximal erythrocyte-labeling with Cr^{51} in 18 patients in whom hematocrit values varied from 16.4 to 49.6 per cent, corrected in the manner explained by Owen and Power.[9] An approximately linear relationship seems to hold, with poorer labeling as the proportion of erythrocytes diminishes.

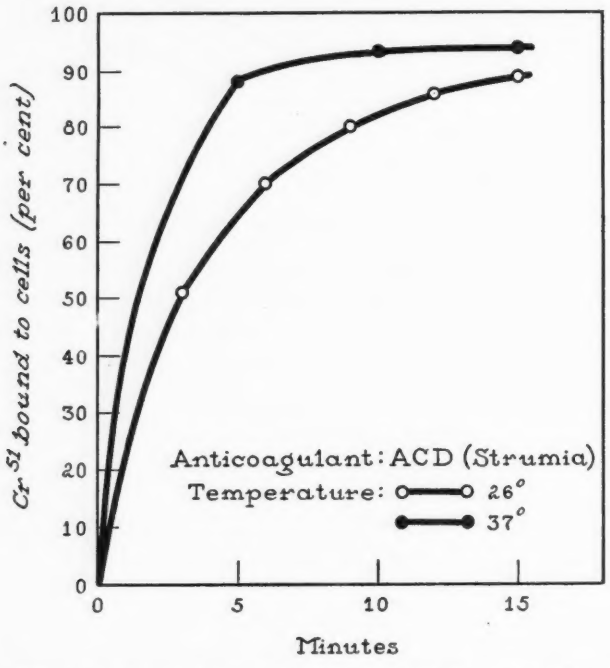

Fig. 4.—Effect of temperature on efficiency of labeling of erythrocytes with Cr^{51}.

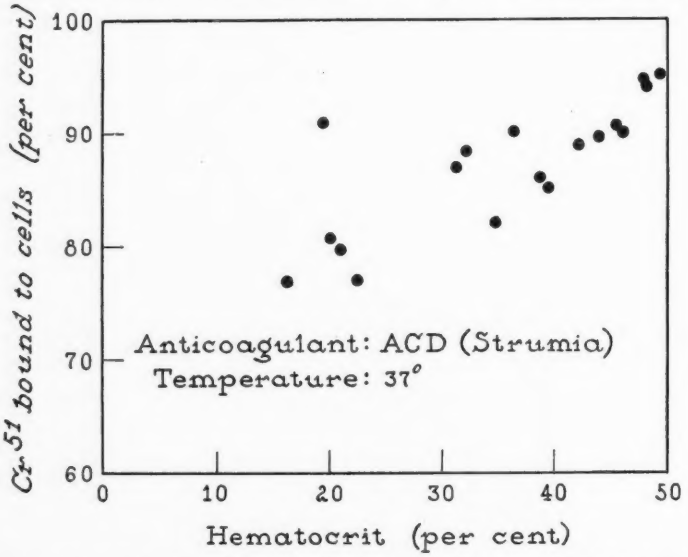

Fig. 5.—Efficiency of erythrocyte-labeling with Cr^{51} in 18 cases in which the hematocrit values varied from 16.4 to 49.6 per cent.

5. *Effect of Order of Addition of Components on Cr^{51}-Labeling.*—To evaluate the effect of the Strumia ACD solution on chromate,[51] the radiochromium solution was added to the ACD solution (a) after the whole blood (control), (b) five hours before the blood was added, and (c) twenty days before the addition of blood. The mixture of chromate[51] and ACD solution for five hours had no observable effect on labeling, but in twenty days the erythrocytes were able to bind only about 5 per cent of the Cr^{51} as compared with the control.

DISCUSSION

Labeling of erythrocytes with Cr^{51} never reaches 100 per cent when the Cr^{51} is added to whole blood. Presumably this is because a small amount of chromate ion can be bound directly to the plasma or, more important, plasmatic reducing substances convert the chromate to the chromic ion and the latter is bound to the plasma proteins. Only the chromate ion seems capable of penetrating the erythrocyte membrane. The longer the exposure of the chromate ion to the reducing substances of the plasma, the less the cellular labeling. This can be demonstrated by mixing plasma with radiochromate for varying periods of time before the erythrocytes are added (Table I).

TABLE I. REDUCED EFFICIENCY IN LABELING OF ERYTHROCYTES WHEN THE CHROMATE[51] SOLUTION IS PREMIXED WITH PLASMA

MIXING TIME OF SODIUM CHROMATE-CR^{51} AND PLASMA	PER CENT CR^{51} BOUND TO ERYTHROCYTES
1 minute	80.0
1 hour	57.4
2 hours	54.3
3 hours	51.4
4 hours	50.5

Whole blood was mixed with Strumia's citrate-dextrose anticoagulant and the plasma obtained by centrifugation. To the plasma was added the radiochromate solution for the specified periods. Finally, a constant volume of erythrocytes was added and labeling was permitted to progress for fifteen minutes at 26° C. The Cr^{51} in the cells, after washing with saline solution, is recorded as the per cent of Cr^{51} originally added to the plasma.

Factors which tend to increase erythrocyte labeling include the use of a citrate-dextrose solution (particularly the Strumia type), a temperature of 37° C., and a high hematocrit value. However, the patient's hematocrit value cannot be controlled, and labeling at room temperature for fifteen minutes proved to be almost as effective as at 37° C. Since ACD solutions do not materially reduce the chromate ion within the first few hours, it is possible to add the Cr^{51} to the ACD solution prior to addition of the whole blood.

Our routine method of labeling erythrocytes with Cr^{51}-sodium chromate, for studies of survival of erythrocytes or quantitation of bleeding, is based on the foregoing findings. Ten milliliters of Strumia's ACD solution is placed in a 100 ml., rubber-capped bottle and is heat-sterilized. When a study is to be conducted in the laboratory, we place about 50 ml. of the patient's venous blood in the bottle and follow this with about 300 μc Cr^{51} (containing less than 500 μg Cr). The mixture is swirled for fifteen minutes at room temperature. A carefully measured volume, usually 50 ml. of the mixture, is returned to the patient. For hospitalized patients we first add the Cr^{51} to the ACD solu-

tion within an hour of the labeling procedure; then at the bedside the blood is added; all is mixed for fifteen minutes and given intravenously to the patient.

For the determination of survival rates of the labeled erythrocytes, Ebaugh and colleagues[4] have recommended the measurement of Cr^{51} per milliliter of cells; that is, $\frac{Cr^{51} \text{ per milliliter whole blood}}{\text{hematocrit value}}$. Strumia and associates[10] have pointed out that this method may be seriously misleading if the patient is generating new cells at a rate significantly different from the rate of destruction. To circumvent this problem, Strumia's group determined the Cr^{51} per milliliter of cells and corrected for changes in the bodily erythrocyte mass as determined by multiple determinations of blood volume.

As an example, assume that, in a given case, aplastic anemia caused an erythrocyte volume of 2 L. to shrink to 1 L., a plasma volume of 3 L. to increase to 4 L., and, therefore, the hematocrit value to fall from 0.4 to 0.2. It may be assumed further that the patient's blood was labeled initially to the extent of 100 cps per milliliter of erythrocytes; that is, 40 cps per milliliter of whole blood initially and 20 cps per milliliter finally (ignoring radioactive decay and elution of Cr^{51} from the cells). According to the basic formula of Ebaugh's group, the apparent value for survival of erythrocytes would be 100 per cent. Taking into account the change in bodily erythrocyte mass, the formula of Strumia's group would be: apparent cell survival $\times \frac{\text{final red-cell mass}}{\text{initial red-cell mass}}$ $= 100$ per cent $\times \frac{1}{2} = 50$ per cent survival, which in this example is correct. Much simpler, though, is the avoidance of blood-volume and hematocrit determinations by measuring the radioactivity of the whole blood; in this case:

$$\frac{20 \text{ cps/ml. blood finally}}{40 \text{ cps/ml. blood initially}} = 50 \text{ per cent.}$$

It can be readily calculated that when the blood volume remains constant the measurement of the Cr^{51} per milliliter of whole blood alone accomplishes the same goal as the tedious method proposed by Strumia's group. This simplification is imperfect if the blood volume varies; that is, changes in erythrocyte mass are not completely compensated for by changes in plasmatic volume, but calculation indicates errors of only a few per cent with any moderate fluctuation of whole-blood volume. If new cells are being formed at approximately the same rate as old cells are being lost or destroyed, all three methods give valid results.

To calculate survival of erythrocytes, we assay the radioactivity of whole (heparinized) blood and plot the radioactivity per milliliter. After the first two to five days the observed values are usually linear when plotted logarithmically against the time in days. There is usually a slightly faster fall in radioactivity during the early period, and this also has been found with the Ashby technique.[11] Values for the first few days, therefore, are ignored unless

the slope is so steep as to indicate severe cellular destruction or loss. The survival value is calculated as the slope of the linear portion of the curve or, more simply, as the half-time of this linear portion.

In the cases of seven normal young adults, the 50 per cent survival time varied between twenty-eight and thirty-seven days. This is in excellent agreement with published values derived from studies in which other labeling techniques and methods of assay were employed.

SUMMARY

1. The kinetics of erythrocyte labeling with Cr^{51} were studied by blocking the reaction at various time intervals with ascorbic acid.

2. The degree of labeling varied with the anticoagulant used, from least to most effective: heparin, sodium citrate, Baxter brand of citrate-dextrose, and the citrate-dextrose preparation of Strumia.

3. Erythrocytes were labeled much more rapidly at 37° C. than at 26° C. in citrate-dextrose solution, but after fifteen minutes the difference was slight with the Strumia preparation.

4. The less the hematocrit value, the less efficient was the Cr^{51}-labeling.

5. These findings are applied in labeling to determine survival of erythrocytes or quantitation of bleeding into the alimentary tract.

REFERENCES

1. Gray, S. J., and Sterling, K.: The Tagging of Red Cells and Plasma Proteins With Radioactive Chromium, J. Clin. Invest. **29**: 1604-1613, 1950.
2. Read, R. C.: Studies of Red-cell Volume and Turnover Using Radiochromium: Description of New "Closed" Method of Red-cell-volume Measurement, New England J. Med. **250**: 1021-1027, 1954.
3. Weinstein, I. M., and LeRoy, G. V.: Radioactive Sodium Chromate for the Study of Survival of Blood Cells. II. The Rate of Hemolysis in Certain Hematologic Disorders, J. LAB. & CLIN. MED. **42**: 368-376, 1953.
4. Ebaugh, F. G., Jr., Emerson, C. P., and Ross, J. F.: The Use of Radioactive $Chromium^{51}$ as an Erythrocyte Tagging Agent for the Determination of Red Cell Survival In Vivo, J. Clin. Invest. **32**: 1260-1276, 1953.
5. Owen, C. A., Jr., Bollman, J. L., and Grindlay, J. H.: Radiochromium-labeled Erythrocytes for the Detection of Gastrointestinal Hemorrhage, J. LAB. & CLIN. MED. **44**: 238-245, 1954.
6. Owen, C. A., Jr., Cooper, M., Grindlay, J. H., and Bollman, J. L.: Quantitative Measurement of Bleeding From Alimentary Tract by Use of Radio-chromium-labeled Erythrocytes, Proc. Surg., Forum, Clin. Congress Am. Coll. Surgeons **5**: 663-667, 1954.
7. Cooper, M., Stroebel, C. F., Stickney, J. M., and Owen, C. A., Jr.: Radiochromium in the Study of Red-cell Survival and Gastrointestinal Bleeding, Fed. Proc. **14**: 31, 1955.
8. Strumia, M. M.: Methods of Blood Preservation in General and Preparation, Preservation and Use of Red Cell Suspensions, Am. J. Clin. Path. **24**: 260-265, 1954.
9. Owen, C. A., Jr., and Power, M. H.: Intercellular Plasma of Centrifuged Human Erythrocytes as Measured by Means of $Iodo^{131}$-Albumin, J. Appl. Physiol. **5**: 323-329, 1953.
10. Strumia, M. M., Taylor, L., Sample, A. B., Colwell, L. S., and Dugan, A.: Uses and Limitations of Survival Studies of Erythrocytes Tagged With Cr^{51}, Blood **10**: 429-440, 1955.
11. Evans, R. L., Amatuzio, D. S., and Ebert, R. V.: Red Blood Cell Studies: Ashby Curves, Science **115**: 572-573, 1952.

APPLICATION OF THE Cr^{51} TECHNIQUE TO THE STUDY OF EXPERIMENTAL HEMOLYSIS IN THE DOG

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STUDIES of red cell survival in the experimental animal have been limited by the difficulty in obtaining antisera adequate for quantitative differential agglutination.[1, 2] When adequate sera are available, the measurement of surviving inagglutinable cells is tedious, time consuming, and subject to considerable error. Isoimmunization has also presented a problem. In our experience, evidence for isoimmunization has been found following 8 of 33 transfusions where donor and recipients had been cross-matched and were compatible for the canine antigens described by Young and associates.[3] The in vivo labeling of red blood cells with isotopes such as C^{14},[4, 5] N^{15},[6] or Fe^{55} or Fe^{59},[7, 8, 9] which eliminates the necessity of transfusion, has been limited due to significant reutilization of the isotope or the technical difficulties encountered in the measurement of activity.

Recently Ebaugh and associates,[10] and Nechles[11] have demonstrated that radioactive chromium^{51} can be used for estimation of red cell survival in humans. The technique employed permits autotransfusion and there is no significant reutilization of the isotope. Moreover, since Cr^{51} is a γ emitter, the accurate measurement of activity does not present a problem. The one disadvantage to the chromium method is that the loss of activity is greater than can be attributed to cell death alone. In normal males, Ebaugh[10] observed that this excessive loss was an exponential function with a half life of 77 ± 12 days or an average daily rate of loss of 0.9 per cent. A similar figure was reported by Nechles,[11] based on in vitro as well as in vivo studies. A leakage, or elution, of chromium from the surviving cells appears to be the most likely explanation for this phenomenon.

In the present report, data are to be presented on the applicability and limitations of this method for estimation of red cell survival in an experimental animal, the dog.

METHODS AND MATERIALS

To establish the validity of the technique, simultaneous measurements of red cell survival by Cr^{51} and differential agglutination techniques was made. Two hundred fifty milliliters of fresh, normal serologically identifiable cells was collected in ACD,* chromated, as described below, and transfused into suitable recipients weighing approximately 15 kg. When red cell survival was estimated with Cr^{51} alone, following auto- or homotransfusions, only 30 to 50 ml. of blood was required. The red cell mass of all recipients was kept constant by removing immediately prior to transfusion a volume of blood equivalent to

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*Acid citrate dextrose N.I.H. formula B.

that which they were to receive. When survival by differential agglutination was measured, the donors were A-negative and recipients A-positive.[3] Compatibility was established for the canine agglutinogens B, C, D, E, and F,[3] and the animals were cross-matched with the indirect globulin technique. The technique of differential agglutination has been previously described.[2]

Red cells were tagged with Cr^{51} by introducing 0.2 to 0.5 ml. of an aqueous solution of radioactive sodium chromate, $Na_2Cr^{51}O_4$, into fresh whole blood, collected in ACD. Five to fifteen micrograms of chromium metal with a specific activity of 0.7 to 2.5 mc. per milligram was added per milliliter of cells. The blood was mixed immediately and incubated for one hour at room temperature. During incubation, the blood was mixed by hand every five to ten minutes. Determination of the activity of plasma and red cells immediately following incubation showed that 85 to 90 per cent of the Cr^{51} had been taken up by the red cells. Removal of plasma prior to transfusion was not considered necessary since Ebaugh[10] noted that no more than 12 per cent of the plasma radioactivity, or about 1 per cent of the total injected activity, became attached to the recipients' red cells following transfusion. The Cr^{51} activity in the recipient was determined twice a week. From 1 to 3 ml. of venous blood was collected in oxalate and measured; the capillary hematocrit was determined by the method of Strumia,[12] the cells hemolyzed with saponin, and the volume brought to 6 ml. with distilled water. On the day of transfusion and the following day the blood was washed twice with isotonic saline to remove residual plasma radioactivity. After that, the plasma radioactivity was insignificant. Samples were counted in a shielded well-type scintillation counter with an over-all efficiency of approximately 3 per cent for Cr^{51} and a background of 200 to 210 C P M. Twelve thousand, eight hundred counts were measured in duplicate and corrected for physical decay; activity was then calculated as C P M per milliliter of packed cells.

Calculation of "Elution" Rates.—The destruction of normal red cells has been considered by most observers to be due to death from aging or senescence alone.[13, 14] Where this is the only source of destruction, the number of transfused cells remaining t days following transfusion is given by:

$$(1)\quad N_t = N_o\left(\frac{T-t}{T}\right),$$

where: N_t = number of cells surviving to time t,
N_o = number of cells transfused,
T = potential life span in days.

If there is elution of chromium from each red cell and if this elution is a constant per cent of the amount of chromium remaining in the red cell, the amount of chromium still remaining in the red cell at any time, t, would be proportional to:

(2) e^{-kt}, where k is the constant rate of loss for Cr^{51}.

Thus Cr^{51} activity of tagged red cells reflects the loss due to cell death and chromium leakage and can be given as the product of the two expressions above:

$$(3)\quad N_o\left(\frac{T-t}{T}\right)e^{-kt}.$$

From expressions (1) and (3), it is possible to compute the constant of chromium elution, k:

$$(4)\quad \frac{A}{C} = \frac{N_o\left(\frac{T-t}{T}\right)}{N_o\left(\frac{T-t}{T}\right)e^{-kt}} = e^{-kt}$$

where A = per cent of the original cells remaining at time t and C = per cent of the original Cr^{51} activity remaining at time t.

$$(5)\quad \log_e\left(\frac{A}{C}\right) = kt;\quad k = \frac{\log_e\left(\frac{A}{C}\right)}{t}.$$

If there is hemolysis present, and it is assumed that the hemolytic process is a constant random process (beginning at the time of the transfusion), then the curve found by the differential agglutination technique is described by:

$$(6)\quad N_t = N_o\left(\frac{T-t}{T}\right)e^{-bt},$$

where b = the rate of constant "random loss," or hemolytic destruction.

In the Cr^{51} measurements, assuming that the hemolytic process in no way changes the constant chromium elution, the curve of apparent survival can be described by:

$$(7)\quad N_t = N_o \left(\frac{T-t}{T}\right) e^{-(b+k)t}.$$

The constant of chromium elution can then be calculated exactly as before:

$$(8)\quad \frac{A}{C} = \frac{N_o \left(\frac{T-t}{T}\right) e^{-bt}}{N_o \left(\frac{T-t}{T}\right) e^{-(b+k)t.}} = e^{kt}$$

and a result identical with (5), above, is found:

$$k = \frac{\log_e \left(\frac{A}{C}\right)}{t}.$$

This means that if k is constant, the ratio of surviving cells to Cr^{51} activity plotted against time t will give a straight line when plotted on semilogarithmic paper. The slope of the line will be proportional to k.

If there have been a series of simultaneous differential agglutination and Cr^{51} readings over a time t, following transfusion, k can be estimated (by least squares, assuming that all points are equally well determined) from:

$$k_t = \frac{\Sigma\left(\log_e \frac{A}{C}\right)_i t_i}{\Sigma t_i^2}$$

where t_i is the i^{th} day following transfusion and k_t is the estimate of k for the first t days following transfusion.

When values for differential agglutination are not available, as in the case of autotransfusion of Cr^{51}-tagged cells or when cells are tagged in vivo, a direct measurement of Cr^{51} elution cannot be made. An estimate, however, may be obtained from a "theoretical curve of senescence." This is constructed by drawing a straight line from the value at time 0 to the point of extinction. This theoretically represents the loss of cells due to senescence. In the absence of hemolysis in either donor or recipient, values obtained from this curve should be identical with the true values for differential agglutination. The rate of Cr^{51} elution, k, is computed as before (5). In the presence of hemolysis, the values for k obtained in this fashion will include loss of Cr^{51} from both elution and hemolysis.

For k to be a constant and the ratio of differential agglutination to Cr^{51} (A/C) a straight line on semilogarithmic paper, certain conditions must be met: (1) a constant chromium elution; (2) a constant random loss of cells if there is loss in addition to that from senescence and if values for differential agglutination have not been measured; and (3) independence of Cr^{51} elution from damage or loss of red cells. When these assumptions are not met, k will not be a constant and the ratio of differential agglutination to Cr^{51} will not plot as a straight line on semilogarithmic paper.

In this paper, we are concerned primarily with those cases in which the assumptions are met. A separate communication will deal with cases in which the assumptions apparently have not been met and the k value is not a constant.

RESULTS

Simultaneous measurement of the survival of transfused normal erythrocytes by Cr^{51} and differential agglutination is illustrated in Fig. 1. The point at which Cr^{51} activity was no longer detectable coincided in this and other transfusions with the disappearance of serologically identifiable cells. This indicates that Cr^{51} activity is a measure of life span and that detectable reutilization of chromium does not occur. As would be anticipated from the observations in humans,[10, 11] the loss of Cr^{51} activity could not be accounted for by the death of transfused cells alone. The loss of Cr^{51} activity in excess of

that from cell death in normal dogs appeared to be an exponential function (Table I, Fig. 2) with the loss constant (k) varying from 1 to 2 per cent in normal dogs. Similar values were obtained following homotransfusions of 20 to 50 ml. of normal blood where the calculation of elution rate was based on a "theoretical senescence curve" (Figs. 2, 3, Table I).

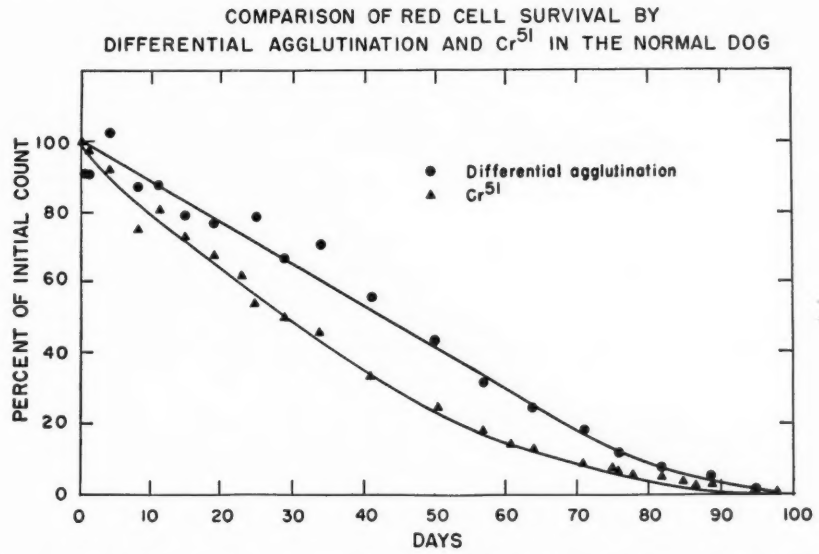

Fig. 1.—Posttransfusion survival of normal erythrocytes determined by Cr^{51} and differential agglutination.

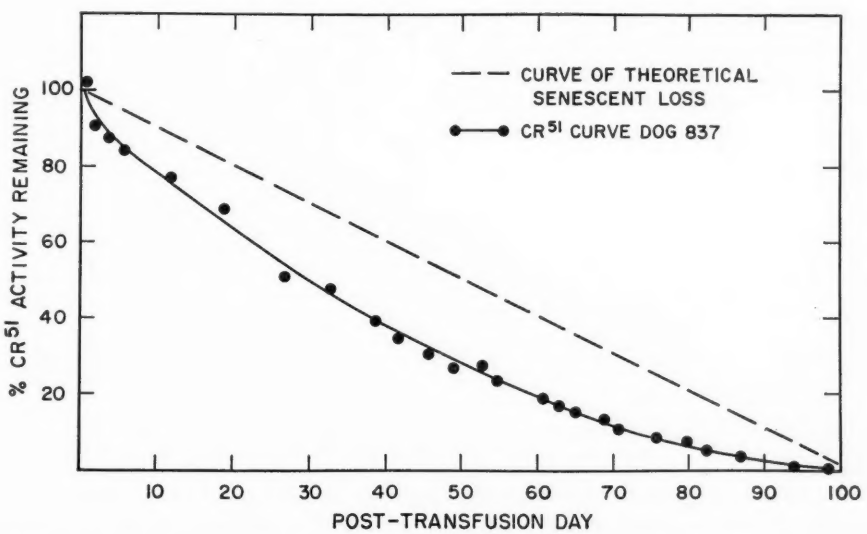

Fig. 2.—Posttransfusion survival of normal erythrocytes determined by Cr^{51}. Broken line indicates curve of theoretical senescence.

Nechles and co-workers[11] have suggested that Cr^{51} data be expressed in terms of an apparent half time of Cr^{51} loss. The Cr^{51} values are plotted on semilogarithmic paper and the time at which 50 per cent of the original

activity remains is called the apparent half time. In normal dogs, this value varied from 21 to 30 days (Fig. 4). This figure includes loss of Cr^{51} activity due to elution, cell death from senescence, and, when present, loss of red cells due to hemolysis or bleeding. Except in the presence of marked random loss of cells (Fig. 4), the curve does not plot as a straight line on semilogarithmic paper.

TABLE I. CALCULATED CHROMIUM ELUTION

DAYS AFTER TRANSFUSION	RATE ± STANDARD ERROR			
	DOG 997	DOG 837	DOG 648	DOG 678
1-10	.0187 ± .0024	.0111 ± .0038	.0169 ± .0023	.0197 ± .014
11-20	.0193 ± .0056		.0185 ± .0089	.0108 ± .005
21-30	.0158 ± .0039	.0110 ± .0068	.0173 ± .0024	
31-40				.013 ± .0003
41-50	.0161 ± .0039	.0127 ± .0034	.0147 ± .0055	
More than 51				.0103 ± .0084
Total (Day 1 to last observation)	.0162 ± .0017	.0123 ± .0014	.0155 ± .0007	.0114 ± .0011

Dog 678 values computed from observed Cr^{51} and differential agglutination. Dog 997 (autotransfusion), Dog 837 (homotransfusion), Dog 648 (in vivo tag) were computed using observed Cr^{51} values and a theoretical senescence curve (see text).

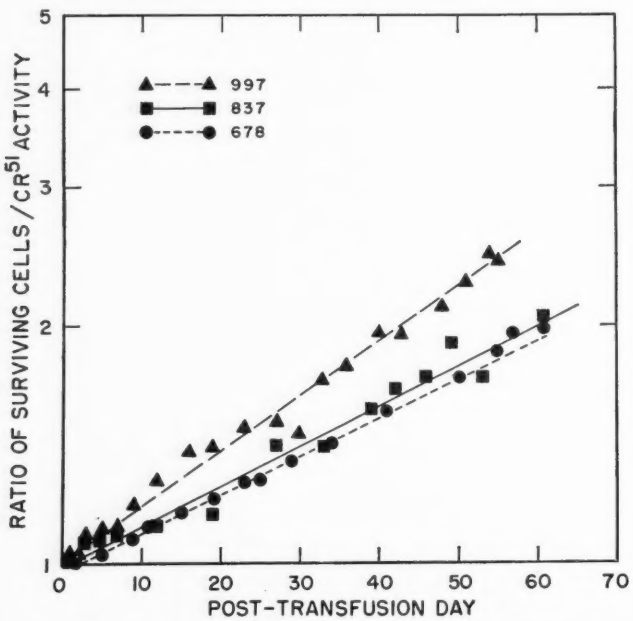

Fig. 3.—Cr^{51} elution plotted on a *semilogarithmic* scale. Dogs 678 and 837 received homotransfusion, and dog 997, autotransfusion.

During the course of these studies of red cell survival, 8 of 33 dogs developed isoantibodies even though compatible for the known canine antigens. At times, the in vitro demonstration of such antibodies has not been possible. In one such case, rapid hemolysis of Cr^{51}-tagged cells occurred on the seventh to tenth day in 4 different recipients when transfused with blood from the same donor. An antibody could not be detected in vitro by the indirect globulin technique. There was no evidence for blood loss or hemolysis of the re-

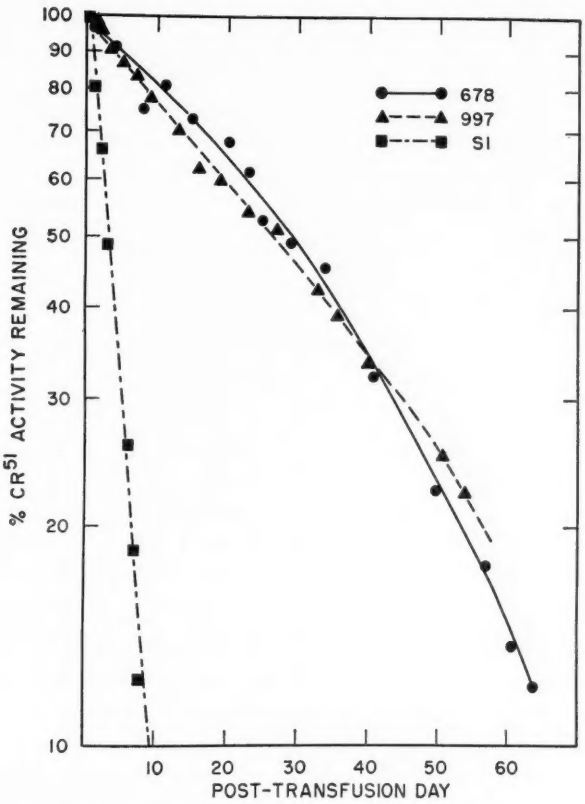

Fig. 4.—Posttransfusion survival of Cr^{51} red cells plotted on a *semilogarithmic* scale. Dogs 678 and 997 show normal survival. Dog S1 had a severe hemolytic syndrome due to a lucite ball-valve prosthesis in the vascular system.[23]

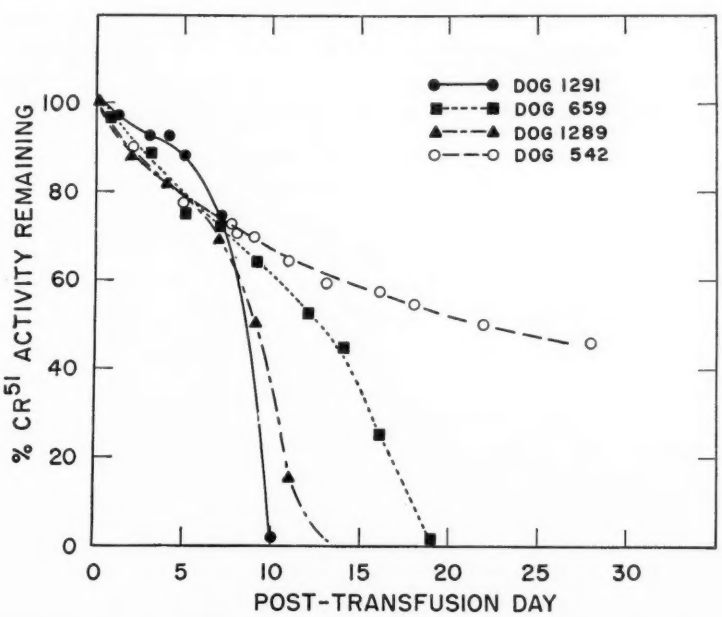

Fig. 5.—Survival curves showing apparent isoimmunization in dogs 1291 and 1289 following transfusion of cells from dog 542. Antibodies were not demonstrable in vitro. The curve of dog 542 represents survival of these donor cells following autotransfusion. The survival curve of a dog (659) in whom isoimmunization to A_1 was demonstrated in vitro is shown for comparison.

cipients' own cells. Survival of the donor red cells following autotransfusion was not characterized by the same rapid hemolysis (Fig. 5). It seems reasonable, therefore, to assume that isoimmunization occurred even though the antibody was not demonstrable in vitro with the present techniques. In 2 other cases when rapid hemolysis developed between the twentieth and thirtieth posttransfusion day, an antibody could not be demonstrated in vitro. In all of these transfusions, the type of curve resulting was similar to that where antibodies could be demonstrated (Fig. 5).

In one dog, random destruction of serologically identifiable transfused cells was evident by both Cr^{51} and differential agglutination techniques. The rate of random destruction determined by differential agglutination was 1.5 per cent per day, and the apparent Cr^{51} half time, sixteen days. While the increased rate of destruction may have been on an immunologic basis, it seems unlikely that it was due to isoimmunization since the increased rate of destruction was evident immediately after transfusion and there was no change in the rate of destruction during the course of the survival study. In this dog, determination of the elution of Cr^{51}, as previously described, indicated an initially increased elution rate (k) which was not constant but progressively declined over the period of study.

Red cell survivals estimated by autotransfusion of Cr^{51}-tagged cells closely paralleled those following homotransfusion. The life span in these studies varied from 90 to 120 days, and the apparent Cr^{51} half time fell within the range of 21 to 30 days. Since the values for cell death could not be directly determined by differential agglutination, the rate of Cr^{51} elution was calculated using a theoretical senescence curve as previously described. In 5 apparently normal dogs the elution computed in this fashion corresponded to the values of 1 to 2 per cent (Table I) obtained when elution was directly determined by simultaneous Cr^{51} and differential agglutination measurements.

It is conceivable that the rate of Cr^{51} elution might depend on the concentration of chromium metal per unit of cells. To reduce significantly the concentration of chromium, it would be necessary to use large volumes of blood, which would be technically difficult to handle, or reduce the counting accuracy by decreasing the activity per unit of cells. If in vivo tagging of red cells could be accomplished and chromium distributed throughout the red cell mass, significantly lower concentrations of metal per unit of cells could be achieved without sacrificing accuracy.

Red cells were tagged in vivo by injecting over a five-minute period 800 μc of Cr^{51} into 15 kg. dogs. Approximately 10 per cent of the injected Cr^{51} was fixed to the red cell within the first twenty-four hours. There did not appear to be any further uptake of Cr^{51}, although there continued to be radioactivity in the plasma over a four to six week period, necessitating washing of the samples. Presumably the plasma Cr^{51} after the initial period is in a chemical form which is not taken up by the red cell. Assuming that the Cr^{51} fixed to the red cells was distributed evenly throughout the red cell mass, the concentration of chromium metal was of the order of 0.15 μg per milliliter of

red cells. In 3 dogs, the apparent half time and the apparent Cr^{51} elution computed with a theoretical senescence curve were similar to those where 5 to 10 μg of chromium per milliliter of cells was used (Table I).

DISCUSSION

The present studies indicate the applicability of the Cr^{51} technique in the study of experimental hemolysis in the dog. The life span of normal canine red cells as measured by this technique varies from 90 to 120 days, which is in agreement with the results obtained with differential agglutination, Fe and N^{15} or C^{14} glycine.[1, 4, 9, 15, 16, 17, 18] As in man,[10, 11] decrease in Cr^{51} activity is not a direct measure of cell destruction, since it was demonstrated by simultaneous differential agglutination and Cr^{51} estimates that the loss of Cr^{51} is greater than would be anticipated from cell death alone. The excess loss of Cr^{51} was an exponential function in normal dogs with a constant rate of loss amounting to 1 to 2 per cent per day. The determination that this loss was a constant is compatible with the hypothesis that Cr^{51} is not firmly bound to the red cell and as a consequence there is a leakage or elution of a fixed percentage of Cr^{51} from the surviving cells.[10, 11] These observations are not compatible with selective tagging or selective destruction, two other possibilities that have been advanced.[10] Selective tagging if due to a greater uptake of Cr^{51} by older cells would lead to a progressively decreasing rather than a constant percentage of Cr^{51} loss throughout the period of study. The converse would be true if younger cells were selectively tagged. Selective destruction, the more heavily tagged cells being preferentially destroyed, would also produce a decreasing rather than a constant percentage of Cr^{51} loss. Elution rates computed with a theoretical senescence curve, drawn from the value at time 0 to the point of extinction, in dogs receiving small homotransfusions, autotransfusions or after in vivo tagging of red cells, gave values of 1 to 2 per cent which were comparable to those measured directly by simultaneous estimates of differential agglutination and $Cr.^{51}$ Elution rates were unaffected by alteration of chromium metal concentration between 0.15 and 10 μg per milliliter of cells. A similar experience in man has been recently reported by Mollison[19] using high specific activity Cr^{51}.

The variability of the elution rate in normal dogs from 1 to 2 per cent limits the usefulness of this procedure as a truly quantitative measure of cell survival. If a mean elution rate is used to correct a given Cr^{51} curve, either an overestimate or underestimate of the rate of cell destruction might result. This could suggest hemolysis where none existed or mask the presence of hemolysis. Consequently, detection of minor degrees of hemolysis is difficult with this technique.

The Cr^{51} curve may, at times, be misleading, because of an abnormal distribution in the age of tagged cells. Normally it is assumed that there are equal numbers of cells of all ages present in the transfused blood. Such a distribution of cells gives a straight-line senescence curve when determined by differential agglutination. When there has been a continued slow random loss, over an extended period of time, the age distribution of cells becomes distorted. There

will be fewer old cells and many more young cells than are normally present. If random loss continues after transfusion of such blood, as would be the case after autotransfusion, the majority of Cr^{51} loss will be due to elution and random loss. Relatively few cells will be lost due to senescence. As a consequence of the decrease in the number of cells lost from senescence, the Cr^{51} curve following autotransfusion of an abnormally distributed population of cells may fall within the normal range. If following autotransfusion of an abnormally distributed cell population there is a daily loss of Cr^{51} of 1.2 per cent from elution, 1.2 per cent from hemolysis, and 0.2 per cent from senescence, the resultant curve would be indistinguishable from the range of normals where there is a daily loss of 1 to 2 per cent of Cr^{51} activity from elution and 1 per cent from senescence. These limitations apply only when the rate of hemolysis is relatively slow.

Neehles and associates[11] have suggested that Cr^{51} estimates of cell survival be expressed as the apparent half time of Cr^{51} activity. This value varied in the dog from twenty-one to thirty days. The apparent half time is obtained by plotting the Cr^{51} data on semilogarithmic paper. This curve in normals does not represent a single exponential but is the product of a linear curve due to loss of cells from senescence and an exponential curve due to Cr^{51} elution. If cell loss from hemolysis or bleeding is present, these values are also included. As a consequence, the curve is not a straight line on semilogarithmic paper except when there is marked random loss, in which case the linear component representing death from senescence is negligible (Fig. 4). Consequently, the time for the Cr^{51} concentration to decrease from 100 per cent to 50 per cent is normally longer than the time in which the concentration decreases from 50 per cent to 25 per cent. The "apparent half time," then, is not an absolute figure in the usual sense of a physical or biologic half life. The term, however, is a convenient means of expression of Cr^{51} data and appears particularly useful in studies where there is marked shortening of red cell survival. It should also be pointed out that this method of expressing the data does not alter the basic limitation in detection of minor degrees of hemolysis and does not include an estimate of the potential life span.

In this technique, as well as in other existing methods, the data are usually calculated in terms of activity per milliliter of packed cells. The validity of such data is based on the assumption that the red cell mass remains constant. Where production rates are greater than the rate of destruction, there is a dilution of chromated cells and one overestimates the rate of destruction. Conversely, when the rate of destruction is in excess of the rate of production, one will underestimate the rate of cell death. In instances where it is apparent that the red cell mass is changing, it is preferable to consider the value of Cr^{51} in terms of activity per unit of whole blood. This has the advantage that it is not dependent upon production rates but the disadvantage of wider variations than the values for activity per unit of a constant red cell mass, since the plasma volume and body/venous hematocrit of dogs are subject to wide fluctuations.[20]

Isoimmunization during these studies was a particular problem. Even though donors and recipients were compatible for the known canine agglutinins[3] and had been cross-matched prior to transfusion, 8 of 33 dogs apparently developed isoantibodies following transfusion. The development of hemolytic antibodies in these dogs was evident from a change in the rate of destruction of donor cells from seven to thirty days following transfusion unaccompanied by evidence of blood loss or hemolysis of the recipients' cells. Of particular concern was the inability to demonstrate an antibody in vitro in 6 of these dogs, which meant that these antibodies if present prior to transfusion would not have been detected by cross-matching with the indirect globulin technique.

In one dog where a constant random destruction amounting to 1.5 per cent per day was evident when measured by differential agglutination, the Cr^{51} curve had a shortened apparent half time of sixteen days. In addition, the chromium elution was not an exponential function but was initially greater than normal and progressively decreased over the period of study. As a consequence, the differential agglutination/Cr^{51} ratio plotted as a curved line on semilogarithmic paper. The changing rate of Cr^{51} elution in this dog apparently was a result of damage to the red cells.[21] Other data to be published support this concept and suggest that the presence of a changing rate of elution is a sensitive index of red cell damage.[22]

SUMMARY

The applicability of the Cr^{51} technique for study of experimental hemolysis in the dog has been described. It was determined that there was an elution or leakage of Cr^{51} from the surviving red cells, which varied in normal dogs from 1 to 2 per cent. Apparent chromium half times in normal animals varied between twenty-one and thirty days. These values were not influenced by concentrations of chromium metal between 0.15 and 10 μc. Whereas the chromium method is at present semiquantitative and does not permit detection of minor degrees of hemolysis, it nevertheless appears to be an excellent method for most studies of hemolysis since it is technically simple and accurate and does permit autotransfusion, thereby avoiding the development of isoantibodies and allowing observations on cells in their native environment.

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REFERENCES

1. Swisher, S. N., Izzo, M. J., and Young, L. E.: Post-transfusion Survival of Fresh and Stored Dog Erythrocytes Measured by Differential Agglutination, J. LAB. & CLIN. MED. **41**: 946, 1953.
2. Stohlman, F., Jr.: Red Cell Survival in the Dog Determined by a Method of Differential Agglutination Employing Canine Anti-A Serum, J. LAB. & CLIN. MED. **47**: 83-87, 1956.
3. Young, L. E., Christian, R. M., Ervin, D. M., Swisher, S. N., O'Brien, W. A., Stewart, W. B., and Yuile, C. L.: Erythrocyte-Isoantibody Reactions in Dogs, Proc. Internat. Soc. Hem. 226, 1950.

4. Bale, W. F., Yuile, C. L., De La Vergne, L., Miller, L. L., and Whipple, G. H.: Hemoglobin Labelled by Radioactive Lysine Erythrocyte Life Cycle, J. Exper. Med. **90**: 315, 1949.
5. Berlin, N. I., Lawrence, J. H., and Lee, H. C.: The Life Span of the Red Blood Cell in Chronic Leukemia and Polycythemia, Science **114**: 385, 1951.
6. Shemin, D., and Rittenberg, D.: The Life Span of the Human Red Blood Cell, J. Biol. Chem. **166**: 627, 1947.
7. Ross, J. F., and Chapin, M. A.: Effects of Storage of Citrated Blood on the Survival of Transfused Erythrocytes, J. A. M. A. **123**: 827, 1943.
8. Gibson, J. G. II, Aub, J. C., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., and Sack, T.: The Measurement of Post-transfusion Survival of Preserved Stored Human Erythrocytes by Means of Two Isotopes of Radioactive Iron, J. Clin. Invest. **26**: 704, 1947.
9. Finch, C. A., Wolff, J. A., Rath, C. E., and Fluharty, R. G.: Iron Metabolism, J. Lab. & Clin. Med. **34**: 1480, 1949.
10. Ebaugh, F. G., Jr., Emerson, C. P., and Ross, J. F.: The Use of Radioactive Chromium^{51} as an Erythrocyte Tagging Agent for the Determination of Red Cell Survival In Vivo, J. Clin. Invest. **32**: 1260, 1953.
11. Nechles, T. F., Weinstein, I. M., and Leroy, G. V.: Radioactive Sodium Chromate for the Study of Survival of Red Cells. I: The Effect of Radioactive Sodium Chromate on Red Cells, J. Lab. & Clin. Med. **42**: 358, 1953.
12. Strumia, M. M., Sample, A. B., and Hart, E. D.: An Improved Micro Hematocrit Method, Am. J. Clin. Path. **24**: 1016, 1954.
13. Sheets, R. F., Janney, C. D., Hamilton, H. E., and Degowin, E. L.: Studies With Inagglutinable Erythrocytes III: Kinetics of Erythrocyte Destruction in Human Beings, J. Clin. Invest. **30**: 1272, 1951.
14. Dornhorst, A. C.: The Interpretation of Red Cell Survival Curves, Blood **6**: 1284, 1951.
15. Hawkins, W. B., and Whipple, G. H.: Life Cycle of Red Blood Cell in the Dog, Am. J. Physiol. **122**: 418, 1938.
16. Grinstein, M., Kamen, M. D., and Moore, C. V.: The Utilization of Glycine in the Biosynthesis of Hemoglobin, J. Biol. Chem. **179**: 359, 1949.
17. Stewart, W. B., Stewart, J. M., Izzo, M. J., and Young, L. E.: Age as Affecting the Osmotic and Mechanical Fragility of Dog Erythrocytes Tagged With Radioactive Iron, J. Exper. Med. **97**: 147, 1950.
18. Brown, W., Jr., and Eadie, G. S.: An Analytical Study of In Vivo Survival of Limited Populations of Animal Red Blood Cells Tagged With Radio-Iron, J. Gen. Physiol. **36**: 327, 1953.
19. Mollison, P. L., and Veall, N.: The Use of the Isotope Cr^{51} as a Label for Red Cells, Brit. J. Hemat. **1**: 62, 1955.
20. Reeve, A. B., Gregersen, M. I., Allen, T. H., and Sear, H.: Distribution of Cells and Plasma in the Normal and Splenectomized Dog, Am. J. Physiol. **175**: 195, 1953.
21. Stohlman, F., Jr., Schneiderman, M. A., and Brecher, G. B.: The Effect of Red Cell Damage on Cr^{51} Elution, Clin. Res. Proc. **3**: 30, 1955.
22. Stohlman, F., Jr., Schneiderman, M. A., and Brecher, G. B.: To be Published.
23. Stohlman, F., Jr., Sarnoff, S. J., and Case, R. B.: Hemolytic Syndrome Following Insertion of a Lucite Ball-valve Prosthesis in the Vascular System, Clin. Res. Proc. **3**: 96, 1955.

RED CELL SURVIVAL IN THE DOG DETERMINED BY A METHOD OF DIFFERENTIAL AGGLUTINATION EMPLOYING CANINE ANTI-A SERUM

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THE characterization of specific canine hemagglutinins by Young and associates[1, 2] has permitted the investigation of immune hemolytic mechanisms in the dog. The estimation of red cell survival, however, has been limited by the difficulty in obtaining antisera satisfactory for quantitative determinations. Swisher and associates[3] have described a method of differential agglutination with canine anti-D. Unfortunately, D is poorly antigenic and few D-negative dogs will produce an antibody which is suitable for quantitative differential agglutination.[3] The relative frequencies[1] of B (6 per cent), C (99 per cent), and F (99 per cent) agglutinins limit their usefulness in survival studies. The A agglutinogen occurs in approximately 63 per cent of dogs and readily produces a potent agglutinin and frequently a hemolysin.[1, 2] High inagglutinable blanks, however, have been obtained with most anti-A sera, regardless of titer.[3]

The present report describes a method of potentiating the agglutination of A cells by anti-A sera and thus reducing the inagglutinable blanks to suitable levels for differential agglutination.

MATERIAL AND METHODS

Characteristics of the Canine A Agglutinogen and Isoantibody A: The canine isoantibody A acts as a specific agglutinin and hemolysin, is thermostable, fixes complement, and sensitizes red cells for the antiglobulin reaction. Maximum agglutination is achieved only in the presence of fresh canine serum.[2, 4] It has been demonstrated that aggultination requires, in addition to anti-A, complement and a thermostable protein plentiful in bovine serum, which is similar to if not identical with conglutinin.[4, 5] During the investigation of this phenomenon it was observed that the agglutinates formed in the presence of inactivated bovine sera appeared to be more "cohesive" than those present in fresh canine serum alone. Consequently, the possibility of reducing inagglutinable blanks by the addition of bovine serum to an anti-A system was studied.

Young and associates[6] have demonstrated varying degrees of reactivity of type A dog erythrocytes with the anti-A antibody produced by immunizing A negative dogs with A cells. Cells of type A can be divided into subgroups A_1 and A_2 on the basis of the ability of potent anti-A antibodies to hemolyze only A_1 cells in vitro in the presence of complement. A_1 cells suspended in fresh dog plasma are also directly agglutinated by most anti-A sera, whereas A_2 cells may be either agglutinated or only sensitized for the indirect antiglobulin reaction by anti-A in the same system. One example of an antibody reacting only with A_1 cells has been found in a dog of type A_2 immunized with A_1 cells.[6] Aside from these clear-cut subgroups, there appears to be a wide variation in the agglutination of individual A cells (either A_1 or A_2) by a given A serum. This has been shown to be independent of the serum enhancing (conglutinin) mechanisms.

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Production and Preparation of Serum: Anti-A serum was produced by multiple small transfusions (5 c.c.) of A+ blood into A-negative recipients. A dog which developed a high agglutinin (1:512) and low hemolysin titer was bled 250 c.c.; the serum was seperated, inactivated, and stored in multiple small aliquots at -20° C. This serum was used throughout the study. Bovine serum was inactivated at 56° C. for thirty minutes and absorbed three times with equal volumes of washed, packed, pooled canine erythrocytes at room temperature. The sera stored in small aliquots remained potent over a 9-month period. Fresh canine serum was obtained on the day of use.

Method of Differential Agglutination: To insure optimal activity of complement in the anti-A system, veronal-bicarbonate buffered saline with added Mg++ and Ca++ at pH 7.4 was used as a diluent. Twenty cubic millimeters[3] of blood was withdrawn with a hemoglobin pipette from the hub of a needle inserted in the antecubital vein. The pipette was discharged into 2 c.c. of buffered saline. At this dilution, coagulation did not present a problem. The total red cell count was obtained by diluting 0.18 c.c. aliquots of the 1:100 red cell suspension with an equal volume of buffered saline, agitating the mixture, filling each of six chambers with a large bore capillary, and counting in routine fashion. Approximately 3,500 cells were enumerated. To determine the inagglutinable count, 0.18 c.c. of the red cell suspension was incubated at 37° C. for thirty minutes with an equal volume of the "antiserum mixture." The latter consisted of one volume fresh canine serum; one volume inactivated, absorbed bovine serum; and two volumes of anti-A serum. Following incubation, the suspension was spun for one minute at 1,500 r.p.m.; the tube was flicked 30 times, the agglutinates were allowed to settle for fifteen seconds, and the chambers filled, using a large bore capillary. During the early posttransfusion period of a survival study, nonagglutinated cells in 10 squares on each side of 8 chambers were counted; as the number of inagglutinable cells decreased, the number of squares counted was increased until finally 25 small squares were counted. Approximately 2,500 cells were counted, except at the end of the study. For inagglutinable blanks, the entire area of the chamber was counted.

The same hemoglobin pipette and 2 c.c. volumetric pipettes were used for a given dog throughout the period of study. In making dilutions, 0.18 c.c. was delivered from a 0.2 c.c. pipette. The pipette was then rinsed several times with buffered saline, and, using the same pipette, 0.18 c.c. of antisera or buffered saline was added. It has been the experience of others as well as ourselves that canine red cells are particularly sensitive to hemolysis by detergents and cleansing solutions. Consequently, these substances were avoided and only water-washed glassware was used. With these precautions nonspecific hemolysis was avoided.

The inagglutinable count was expressed as inagglutinable cells per 5 small squares, and the blank subtracted from this. This value was divided by the total red count per 5 squares to obtain the percentage of the total count which was inagglutinable. Due to the variability of the plasma volume in the dog,[7] it was felt that this value was more meaningful than the number of inagglutinable cells per cubic millimeter of whole blood.

For the study of posttransfusion survival of serologically identifiable cells, A recipients weighing approximately 15 kilo were used throughout. Donor and recipients, compatible for B, C, D, E, and F hemagglutinins,* were cross-matched with the indirect Coombs technique. Bleeding and transfusion were carried out under Pentothal anesthesia. Immediately prior to transfusion, a volume of blood equal to that to be transfused was withdrawn from the femoral or peripheral vein of the recipient. Blood was collected from the A-negative donor into ACD solution (N.I.H. solution B) from the femoral artery. The transfusions were given by intravenous drip over a period of ten to fifteen minutes.

RESULTS

In all instances where fresh canine serum and anti-A serum were used, the inagglutinable blank counts on A+ dogs were greater than 50,000 and frequently in excess of 100,000 to 200,000. The addition of bovine serum reduced

*The author is indebted to Dr. L. E. Young of the University of Rochester for these antisera.

the blanks to 30,000 or less in approximately 30 per cent of the dogs tested. Incubation at 37° C. appeared to yield results slightly superior to those obtained at room tempature. This presumably is due to the greater activity of complement at this temperature. In some A+ dogs, weak agglutination, ++ to +++ or less, is seen with undiluted serum, and in some the presence of the A agglutinin can only be demonstrated with the indirect globulin technique. In

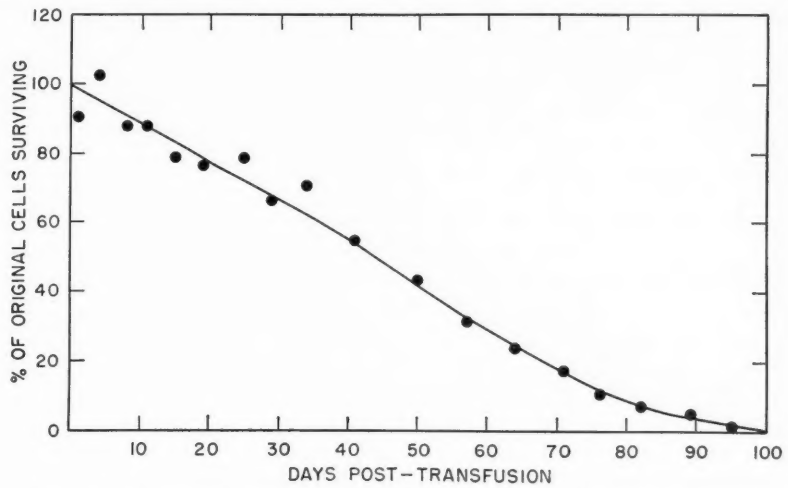

Fig. 1.—Survival of normal canine cells following transfusion into a normal compatible recipient.

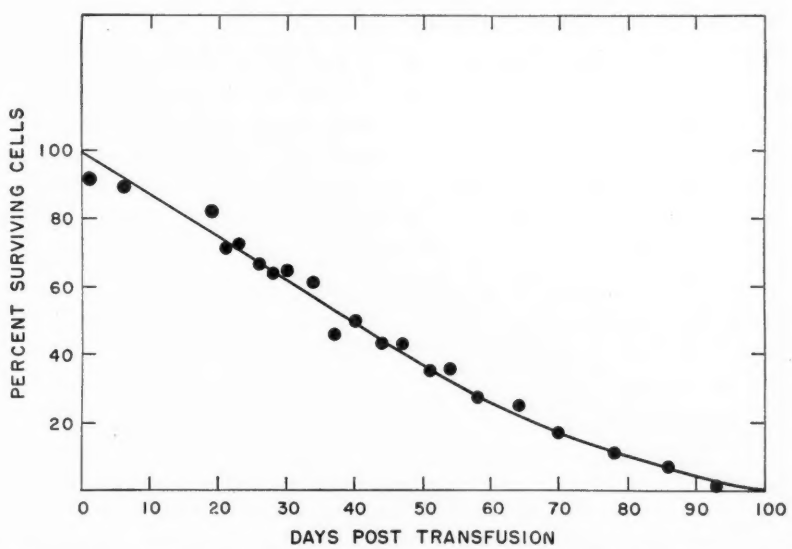

Fig. 2.—Survival of normal canine cells following transfusion into a normal compatible recipient.

such instances, it is apparent from the initial typing of the dogs that they are not satisfactory for survival studies. Dogs with inagglutinable blank counts of 30,000 or less were considered satisfactory for use as recipients in the study of quantitative differential agglutination.

Artificial mixtures of A-positive and A-negative cells were prepared in dilutions which would be encountered during a survival study. Results of such experiments are given in Table I. The agreement between the expected and observed values for inagglutinable counts appeared acceptable when one considers the errors of preparation of such mixtures together with the inherent error of the red cell count.

The loss of serologically identifiable normal cells following transfusion into a normal recipient appeared linear throughout most of the curve (Figs. 1 and 2). In all dogs, there was a tail at the end of the curve (Figs. 1 and 2), which presumably results from the distribution of red cell life spans about a mean. The estimated life span of normal red cells in 4 dogs varied from 90 to 100 days when determined by this method.

TABLE I. DETERMINATION OF INAGGLUTINABLE CELLS IN AN ARTIFICIAL IN TWO IN VITRO MIXTURE IS OF A+ AND A− CELLS

Total A+ cells**	720 ± 12.74*	
Total A− cells	547 ± 9.08*	
Estimated per cent A-negative cells in mixture	7.1 ± 0.3†	4.8 ± 0.2†
Expected inagglutinable count (A-negative cells)	49.8 ± 0.8*	34.2 ± 0.6*
Observed inagglutinable count-blank inagglutinable count	50.7 ± 4.3*	30.5 ± 2.2*
Observed per cent A-negative cells in mixture	7.2 ± 0.6†	4.3 ± 0.3†

*Standard error.
**Per 5 squares.
†95% confidence limits.

DISCUSSION

A method of differential agglutination with canine anti-A, potentiated by bovine serum and complement, has been described. It was not established whether the reduction of inagglutinable counts obtained with bovine serum is due to conglutinin or another heat stable component of bovine serum. Blank inagglutinable counts were reasonably constant over a 6-month period. Satisfactory recovery of inagglutinable cells was obtained with in vitro mixtures of A-negative and A-positive cells.

The posttransfusion survival of normal fresh canine erythrocytes in normal animals approached linearity and gave estimates of life span of 90 to 100 days. Data collected in other dogs in this laboratory using Cr^{51} [8] indicate that the life span of the normal canine erythrocyte may vary from 90 to 130 days. Swisher and associates[9] reported values of 80 to 114 days following the transfusion of fresh or stored canine blood when estimated by a differential agglutination technique employing canine anti-D. With other techniques,[10, 11, 12, 13, 14] including radioiron, C^{14}, and N^{15}, values for red cell life span in the dog of 90 to 135 days have been recorded.

Isoimmunization has also presented a problem in the study of experimental hemolysis. The antigenic structure of the canine red cell is not as well understood as the human; consequently, the likelihood of development of antibodies is great even though the dogs have been typed for the known canine hemagglutinins. Although hemolytic reactions were not observed following trans-

fusions for differential agglutination, other studies in this laboratory have been complicated by the development of hemolytic isoantibodies following transfusion of apparently compatible normal red cells.[8] Of particular concern was the inability in some instances to demonstrate the antibodies in vitro in the presence of unquestionable in vivo evidence of isoimmunization.

SUMMARY

A technique has been described for the quantitative differential agglutination of canine erythrocytes employing anti-A sera which was potentiated by a heat stable fraction of bovine sera. Recovery of A-negative cells from artificial mixtures was satisfactory. Posttransfusion survival of canine erythrocytes indicated a life span of 90 to 100 days.

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REFERENCES

1. Young, L. E., Christian, R. M., Ervin, D. M., Swisher, S. N., O'Brien, W. A., Stewart, W. B., and Yuile, C. L.: Erythrocyte Isoantibody Reactions in the Dog, Proc. Intern. Soc. Hemat. New York, 1950, Grune & Stratton, Inc. 226.
2. Christian, R. M., Ervin, D. M., and Young, L. E.: Observations on the In-vitro Behavior of Dog Isoantibodies, J. Immunol. **66**: 37, 1951.
3. Swisher, S. N., Izzo, M. J., and Young, L. E.: A Method of Quantitative Differential Agglutination of Dog Erythrocytes, J. LAB. & CLIN. MED. **41**: 936, 1953.
4. Stohlman, F., Jr.: Enhancement of Canine Isohemagglutinin A by a Heat Labile Serum Factor, Probably Complement, Proc. Soc. Exper. Biol. & Med. **82**: 563, 1953.
5. Stohlman, F., Jr.: The Nature of Serum Enhancement of the Canine Isohemagglutinin A.: Role of Conglutinin, Clin. Res. Proc. **1**: 67, 1953.
6. Young, L. E., O'Brien, W. A., Swisher, S. N., and Miller, G.: Personal Communication.
7. Reeve, A. B., Gregersen, M. I., Allen, T. H., and Sear, H.: Distribution of Cells and Plasma in the Normal Splenectomized Dog, Am. J. Physiol. **175**: 195, 1953.
8. Stohlman, F., Jr., and Schneiderman, M.: Application of the Cr^{51} Technique to the Study of Experimental Hemolysis in the Dog, J. LAB. & CLIN. MED. **47**: 72-82, 1956.
9. Swisher, S. N., Izzo, M. J., and Young, L. E.: Post Transfusion Survival of Fresh and Stored Dog Erythrocytes Measured by Differential Agglutination, J. LAB. & CLIN. MED. **41**: 946, 1953.
10. Hawkins, W. B., and Whipple, G. H.: Life Cycle of Red Blood Cell in the Dog, Am. J. Physiol. **122**: 418, 1938.
11. Grinstein, M., Kamen, M. D., and Moore, C. V.: The Utilization of Glycine in the Biosynthesis of Hemoglobin, J. Biol. Chem. **179**: 359, 1949.
12. Stewart, W. B., Stewart, J. M., Izzo, M. J., and Young, L. E.: Age as Affecting the Osmotic and Mechanical Fragility of Dog Erythrocytes Tagged with Radioactive Iron, J. Exper. Med. **91**: 147, 1950.
13. Finch, C. A., Wolff, J. A., Rath, C. E., and Fluharty, R. G.: Iron Metabolism; Erythrocyte Iron Turnover, J. LAB. & CLIN. MED. **34**: 1480, 1949.
14. Brown, W., Jr., and Eadie, G. S.: An Analytical Study of In Vivo Survival of Limited Populations of Animal Red Blood Cells Tagged with Radio-Iron, J. Gen. Physiol. **36**: 327, 1953.

SUPPLEMENTAL FOLIC ACID THERAPY IN PERNICIOUS ANEMIA: THE EFFECT ON ERYTHROPOIESIS AND SERUM VITAMIN B_{12} CONCENTRATIONS IN SELECTED CASES

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THE concurrent use of folic acid and of vitamin B_{12} or purified liver extract has been suggested for complete maintenance therapy in certain patients with pernicious anemia on the basis that presumably minimal amounts of both vitamin B_{12} and folic acid are required for normal hematopoiesis.[1-4] It has been repeatedly demonstrated that folic acid alone not only is inadequate treatment for pernicious anemia but also may be responsible for accelerated development of neurological disturbances.[3, 5, 6] In contrast, folic acid has no detectable harmful hematological or neurological effects in normal subjects,[7, 8] and when given together with parenteral vitamin B_{12} or liver extract usually produces no ill effects in patients with pernicious anemia.[9, 10] The study here reported was undertaken in order to assess the need for supplemental folic acid in the maintenance therapy of certain patients with pernicious anemia selected on the basis that they had not achieved normal blood values following prolonged therapy with amounts of vitamin B_{12} seemingly adequate for this purpose in the majority of other patients under observation.

MATERIALS AND METHODS

Thirty-five patients were studied who had been diagnosed as having pernicious anemia on the basis of achlorhydria, macrocytic anemia with abnormal bone marrow, and characteristic hematological response to purified liver extract or vitamin B_{12}. All had been maintained in remission for two years or more with monthly injections either of 15 μg of crystalline vitamin B_{12} or of purified liver extract (Liver Injection U.S.P.) containing 15 μg of vitamin B_{12}. The individuals in this group, representing about one-fifth of the cases of pernicious anemia receiving maintenance therapy in an outpatient clinic at the Boston City Hospital, were chosen because they had had hemoglobin levels of less than 12.5 Gm. per cent for one year or more. In the past, some of these patients had been given larger doses of vitamin B_{12} or of purified liver extract without noticeable benefit.

During the period of this study all the patients were given larger doses of their customary maintenance therapy. Twenty-four of the thirty-five patients selected were thereafter treated every two weeks for up to one year with intramuscular injections of 30 μg of crystalline vitamin B_{12},† and eleven were so treated with Liver Injection U.S.P.,†

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†Kindly supplied by Mr. G. B. Walden of Eli Lilly & Company.

containing, according to the manufacturer, 20 μg of vitamin B_{12}. After each patient had received the increased dosage of vitamin B_{12} as such, or as liver extract, for six months (Period 1), each was given, in addition, 5 mg. of folic acid orally daily throughout a second six-month period (Period 2).

Red cell, hemoglobin, and serum vitamin B_{12} levels were determined from samples of venous blood. Red cell counts were performed using standardized pipettes and counting two chambers filled from two separate pipettes. Hemoglobin determinations were made using a photoelectric colorimeter according to the method of Evelyn.[11] Red cell indices were calculated.[12] Serum vitamin B_{12} concentrations were estimated by a microbiologic technique using *Euglena gracilis* according to a modification[13] of the method of Ross. Strict precautionary measures were observed in order to preclude accidental contamination of samples with vitamin B_{12}. Control red blood cell levels and indices, and serum vitamin B_{12} concentrations were determined at the start of the study. Red cell levels and indices were repeated every two months throughout the study. Assays of serum vitamin B_{12} from blood samples drawn two weeks after the last previous injection of vitamin B_{12} or of liver extract were repeated at six and at twelve months. A change in hemoglobin concentration of more than 10 per cent or in serum vitamin B_{12} concentration of more than 20 per cent was considered to be individually significant. Complete data were obtained on twenty-seven patients of the original group of thirty-five.

RESULTS

Effect on Hemoglobin Levels.—The patients described varying degrees of subjective improvement after beginning to receive the increased dosage of vitamin B_{12} as such, or as liver extract. None experienced symptomatic relapse while taking the additional folic acid. One patient who had glossitis that did not improve after increased vitamin B_{12} therapy described marked regression of lingual symptoms while taking folic acid. The laboratory data obtained are summarized in Table I. Of the twenty-two patients receiving crystalline vitamin B_{12}, only five manifested a significant and sustained increase in hemoglobin six months after starting on the larger doses of vitamin B_{12}. Of the eleven patients who received purified liver extract, none showed significant hemoglobin increments. In no instance did a significant fall of hemoglobin concentration occur during Period 1. Supplemental folic acid therapy during Period 2 caused no significant change, either up or down, in the hemoglobin levels of any patient. One female patient, who proved to have an associated iron deficiency, received folic acid for four months without showing any change. However, after receiving ferrous sulfate, a significant rise in her hemoglobin level occurred.

Effect on Serum Vitamin B_{12} Levels.—The serum vitamin B_{12} levels of the patients on whom this determination was made at the outset, after six, and after twelve months are shown in Table I and Fig. 1. The initial total serum vitamin B_{12} concentration was less than 100 $\mu\mu$g per milliliter in twenty of the thirty-five patients. At the end of Period 1, after each patient had received 30 μg of crystalline vitamin B_{12} or 20 μg of vitamin B_{12} as Liver Injection U.S.P. every two weeks for six months, only two of thirty-one patients who were successfully followed had serum vitamin B_{12} levels of less than 100 $\mu\mu$g per milliliter. At the end of Period 2, after one year of increased vitamin B_{12} therapy and after six months of additional folic acid therapy, none of twenty-nine patients on whom data were obtained had a serum vitamin B_{12} level below 100 $\mu\mu$g per milliliter.

There is, however, no indication that these increments in serum vitamin B_{12} levels were accompanied by increases in hemoglobin or by decreases in mean corpuscular volume (Fig. 2) in the cases studied. As in patients who showed no rise in hemoglobin level, in the five patients in whom a rise occurred

TABLE I. HEMATOLOGICAL AND MICROBIOLOGIC DATA ON THIRTY-FIVE PATIENTS WITH PERNICIOUS ANEMIA WHO RECEIVED INTRAMUSCULAR VITAMIN B_{12} AND SUPPLEMENTAL ORAL FOLIC ACID THERAPY

	INITIAL LEVELS		PERIOD 1: AFTER SIX MONTHS OF INCREASED PARENTERAL VITAMIN B_{12} THERAPY		PERIOD 2: AFTER SIX MONTHS OF SUPPLEMENTAL ORAL FOLIC ACID THERAPY (5 MG. PER DAY)	
PATIENT	HEMOGLOBIN (GM./100 ML.)	TOTAL SERUM VITAMIN B_{12} ($\mu\mu$G/ML.)	HEMOGLOBIN (GM./100 ML.)	TOTAL SERUM VITAMIN B_{12} ($\mu\mu$G/ML.)	HEMOGLOBIN (GM./100 ML.)	TOTAL SERUM VITAMIN B_{12} ($\mu\mu$G/ML.)
			Vitamin B_{12}, 30 μg Every Two Weeks			
1	8.6	1,050	8.8	1,150	9.3	545
2	10.4	106	11.4	305	11.6	330
4	11.0	277	11.5	772	11.8	321
5*	9.2	48	15.3	261	14.3	310
7*	11.4	107	13.1	600	13.3	210
8	11.6	181	12.5	1,360	12.9	482
9	11.5	37	12.5	348	12.9	232
10	12.1	43	13.2	168	—	—
11	11.3	60	—	—	11.1	265
12	11.8	421	12.8	377	12.8	168
13	9.6	52	9.2	143	11.3	144
17	12.0	424	11.7	393	11.8	330
18	12.4	52	12.4	221	12.2	185
19	12.4	207	13.5	900	12.8	295
21	12.7	10	13.0	311	12.8	218
23	11.4	15	11.9	442	11.3	116
24*	13.5	28	14.9	108	15.2	320
25	12.4	810	12.4	570	13.3	240
26*	12.3	44	14.4	325	14.5	253
27	11.0	776	11.2	1,840	—	—
30*	12.4	84	15.3	267	14.6	300
33	11.2	268	11.8	320	11.8	445
34	11.6	53	—	—	—	—
35	11.7	68	12.2	405	12.7	275
			Liver Injection U.S.P. (of Vit. B_{12}, 20 μg) Every Two Weeks			
3	11.8	151	12.8	646	12.6	175
6	11.8	60	12.2	135	12.8	202
14	12.0	186	13.1	360	13.6	238
15	12.4	19	12.8	97	12.5	120
16	11.4	142	11.8	271	12.0	214
20	12.4	98	13.1	—	12.5	208
22	12.6	31	13.5	126	14.2	131
28	12.3	575	12.4	960	13.1	745
29	12.1	46	12.5	—	—	—
31	11.7	76	12.5	194	12.5	—
32	11.0	35	11.8	78	—	—

*Considered to show a significant increase in hemoglobin level.

the serum vitamin B_{12} level returned to a normal range from an initial level of less than 100 $\mu\mu$g per milliliter. Of sixteen patients with initially low levels who were still under observation at the end of Period 1, fourteen had vitamin B_{12} levels within the normal range after six months of increased

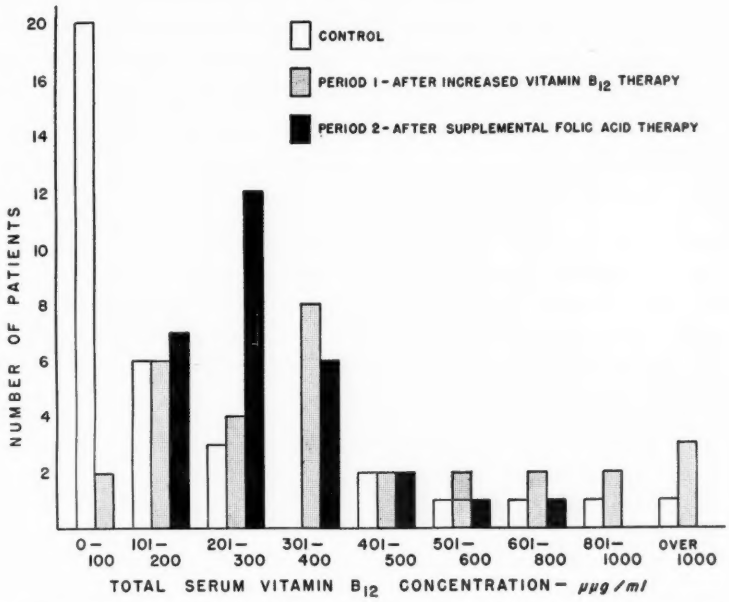

Fig. 1.—Serum vitamin B_{12} levels in relation to period of observation. Patients with pernicious anemia in relapse have serum vitamin B_{12} levels of less that 100 $\mu\mu$g per milliliter. Number of patients on whom estimations were obtained: Control, 35; Period 1, 31; Period 2, 29.

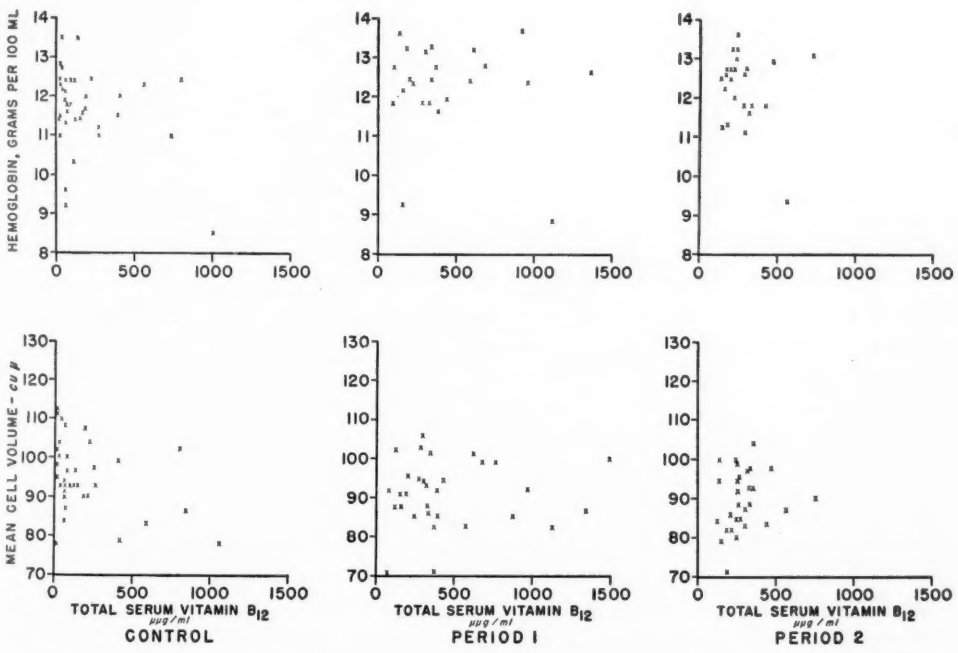

Fig. 2.—Hemoglobin level and mean cell volume in relation to serum vitamin B_{12} level.

vitamin B_{12} therapy. Concerning the remaining two patients, each of whom received a total of 40 μg of vitamin B_{12} per month as liver extract, one had a normal vitamin B_{12} level at the end of Period 2 and the other did not return to the clinic after the end of Period 1. The actual range of serum vitamin B_{12} concentrations varied greatly at the end of each period. Initial control levels ranged from 10 to 1,050 $\mu\mu$g per milliliter with a mean of 198 $\mu\mu$g per milliliter for the entire group. The twenty-four patients who had received crystalline

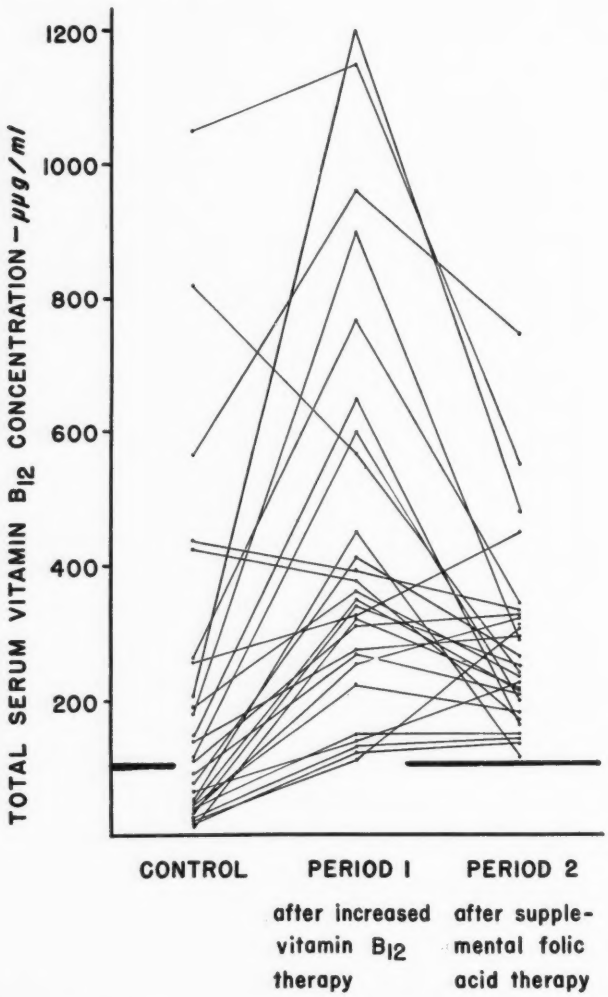

Fig. 3.—Individual serial responses of the serum vitamin B_{12} level in twenty-seven patients with pernicious anemia in relation to period of observation. Note the number of control vitamin B_{12} values below 100 $\mu\mu$g per milliliter.

vitamin B_{12} averaged 225 $\mu\mu$g per milliliter, and the eleven patients who, before these observations, had been receiving purified liver extract averaged 137 $\mu\mu$g. At the end of Period 1, the range for the thirty-one patients on whom data were obtained was 78 to 1,840 $\mu\mu$g per milliliter with a mean of 455. The

subgroup who received crystalline vitamin B_{12} had an average of 527 $\mu\mu$g per milliliter, and the patients who received purified liver extract had an average of 319. Of the thirty-one patients followed six months, twenty-six had increases in serum vitamin B_{12} concentration greater than 20 per cent and one had an increase of between 10 and 20 per cent; two showed no significant change; and two had levels between 10 and 20 per cent below their initial concentration.

Vitamin B_{12} estimations were made on twenty-nine of the original thirty-five patients at the end of Period 2, following six months of folic acid therapy given in addition to the larger vitamin B_{12} dosage. Vitamin B_{12} levels were not obtained before folic acid was started in two of these twenty-nine patients; hence in only twenty-seven patients were vitamin B_{12} levels obtained seriatim. The serum vitamin B_{12} concentration at the end of Period 2 was normal in the two not studied on all three occasions. The average level for the entire group at the end of Period 2 was 276 $\mu\mu$g per milliliter, and the range was 116 to 745. For the twenty-one patients receiving crystalline vitamin B_{12} the average was 285 $\mu\mu$g per milliliter, and for the eight remaining patients on purified liver extract, 254. At the end of Period 2 three patients showed an increase in serum vitamin B_{12} concentration greater than 20 per cent, three an increase of 10 to 20 per cent, and three no change. The most consistent change after administration of supplemental folic acid was the fall in vitamin B_{12} concentration. This occurred in eighteen of the twenty-seven patients on whom serial vitamin B_{12} levels were obtained at control times and at the end of both Periods 1 and 2. In two the vitamin B_{12} level fell 15 to 20 per cent, and in sixteen it fell more than 20 per cent. In this group, fourteen of the twenty patients receiving crystalline vitamin B_{12} and four of the seven patients treated with liver extract showed such a drop. In Fig. 3 are shown the vitamin B_{12} concentrations in twenty-seven individual patients initially and at the end of Periods 1 and 2.

DISCUSSION

The purpose of this study was to evaluate the hematopoietic effectiveness of folic acid as a supplement to vitamin B_{12} therapy in a group of patients with pernicious anemia, who were selected because they had hemoglobin levels in the low normal or mildly anemic range as compared with those of other patients on similar therapy. It was thought that a further rise in hemoglobin subsequent to the addition of folic acid to an optimal dosage schedule of vitamin B_{12} would indicate that folic acid deficiency existed. Chodos,[9] Ross,[2] and Meacham[14] have reported their experiences with vitamin B_{12} and folic acid in the therapy of pernicious anemia. Spray[15] has reported abnormal folic acid utilization in pernicious anemia in relapse. Girdwood[16] has suggested that vitamin B_{12} deficiency may lead to actual or to apparent folic acid depletion and has reported one patient who had deficiency both of folic acid and of vitamin B_{12}. Spies[3] has indicated the possible coexistence of folic acid and vitamin B_{12} deficiency, and Ungley[17] has described two such patients.

Ritz[18] has suggested that suboptimal oral doses of folic acid and vitamin B_{12} have a synergistic action in inducing a response in patients with pernicious anemia in relapse. Reisner[19] has indicated that such therapy in relapse results in enhancement of the hematopoietic effect. It has been well established that folic acid, although not complete therapy for pernicious anemia, does regularly initiate and maintain for varying periods a hematopoietic response. Thus, under various circumstances response to folic acid, although not necessarily indicating a predominant deficiency of folic acid, has been observed in pernicious anemia.

That therapy with folic acid alone can be detrimental, resulting in neurological changes with or without hematopoietic relapse, is similarly well established.[2-6] However, so long as vitamin B_{12} is given together with folic acid, no harmful effects ordinarily ensue.[9, 10] The clinical course of the patients here reported confirms this observation in that none of these patients who received folic acid and vitamin B_{12} manifested any undesirable neurological or hematological change. In fact, the glossitis of one patient improved while she was receiving supplemental folic acid although the hemoglobin level showed no change.

Effect on Hemoglobin Levels.—Prior to the present study patients attending this clinic had been receiving approximately 15 μg of vitamin B_{12} (either as the crystalline vitamin or as purified liver extract) every four weeks for periods up to six years without manifest neurological or hematological relapse. At the end of Period 1, six months after the institution of the increased doses of vitamin B_{12} (20 or 30 μg every two weeks), five of thirty-three patients showed an increase in hemoglobin concentration greater than 10 per cent. This indicates that although maintenance with injections of 15 μg of vitamin B_{12} every four weeks was adequate for normal hemoglobin synthesis in most of these patients (and presumably in the approximately 125 other patients attending this clinic), it was not so for all. It has been suggested[14] that maintenance with 30 μg of vitamin B_{12} per month is probably optimal therapy. Observations of the serum vitamin B_{12} and hemoglobin levels of these clinic patients indicate that 15 μg of vitamin B_{12} every four weeks is suboptimal.

None of the patients here reported had further increment in hemoglobin concentration at the end of Period 2, six months after the institution of supplemental folic acid therapy, thus indicating that concomitant folic acid lack was not a limiting factor in hemoglobin synthesis in these particular patients. Nor was there any decline in hemoglobin concentration to suggest that folic acid might interfere with normal hematopoiesis in pernicious anemia treated with vitamin B_{12}.

Effect on Serum Vitamin B_{12} Levels.—The microbiologic determination of serum vitamin B_{12} has proved to be a useful aid in the diagnosis of the vitamin B_{12} deficient state.[13, 20] Patients with pernicious anemia previously untreated or in relapse have serum vitamin B_{12} levels of less than 100 $\mu\mu$g per milliliter, whereas normal subjects and patients with miscellaneous diseases have serum

concentrations above this value. Twenty patients with pernicious anemia who had been receiving injections of 30 μg of vitamin B_{12} every four weeks were selected at random from the clinic; all but two patients at the end of Period 1 and all patients at the end of Period 2 had serum vitamin B_{12} levels greater than those of patients with untreated pernicious anemia. Serum vitamin B_{12} levels of less than 100 $\mu\mu$g per milliliter initially detected in twenty of the thirty-five patients so tested indicate that treatment with 15 μg of vitamin B_{12} each month is probably suboptimal maintenance therapy in the sense that it is inadequate to replenish body stores. Hence, on this basis we may conclude that the intramuscular injection of 15 μg of vitamin B_{12} every four weeks is inadequate therapy in terms of tissue saturation. Serum vitamin B_{12} levels of patients receiving injections of 30 μg of vitamin B_{12} every four weeks and of those receiving 20 or 30 μg every two weeks for eight to twelve months were above the relapse range. Meacham[14] suggested that 30 μg of vitamin B_{12} every four weeks is adequate and, possibly, optimal maintenance therapy. The serum vitamin B_{12} levels, as indicated previously, are in agreement with the suggestion that 30 μg of vitamin B_{12} monthly is an adequate dosage schedule for most patients.

Neurological relapse in exclusively folic acid-treated pernicious anemia patients occurs in approximately 50 per cent of cases within two years.[2] The rapidity with which relapse develops may correlate with the amount of folic acid given. Clinical observation of patients receiving oral liver preparations in the early days of pernicious anemia therapy suggests that the incidence of neurological relapse was not so frequent as in folic acid-treated patients. Of thirty patients in this clinic who received no antipernicious anemia therapy,[21] none developed neurological relapse in a period of two years, although about half experienced hematological relapse. This suggests that folic acid may have a neuropathic effect, either directly or by altering vitamin B_{12} utilization. The present finding that during Period 2 the daily addition of 5 mg. of folic acid orally to the maintenance therapy with intramuscular vitamin B_{12} was followed in eighteen of twenty-seven patients by a significant drop in serum vitamin B_{12} concentration offers some support to the latter concept. Similarly, studies of the acute effect of large doses of folic acid in monkeys receiving a diet deficient in vitamin B_{12} and folic acid also present suggestive, although not conclusive, evidence that folic acid may cause a fall in serum vitamin B_{12} concentration.[22] Whether this represents a general effect of vitamin imbalance, such as may occur when single members of the vitamin B complex group are administered in the presence of mixed deficiency,[23] or is a specific result of some antagonistic interaction of folic acid and vitamin B_{12}[24] cannot be stated. Nonetheless, it does suggest the theoretical possibility that over a protracted period the combined use of suboptimal amounts of vitamin B_{12} and large amounts of folic acid might result occasionally in the development of neural dysfunction. Chodos[9] described one patient with subacute combined degeneration who improved only after folic acid was omitted from combined folic acid and vitamin

B_{12} therapy, and two patients with pernicious anemia and other organic disease who manifested progression of neurological disturbance while receiving both vitamin B_{12} and folic acid.

SUMMARY

1. Administration of 30 μg of vitamin B_{12} intramuscularly every two weeks increased the hemoglobin levels of only five of a group of twenty-two patients previously receiving 15 μg of crystalline vitamin B_{12} every four weeks. None of eleven patients who received 20 μg of vitamin B_{12} in the form of Liver Injection U.S.P. every two weeks was so benefited.

2. Supplemental folic acid did not alter hemoglobin levels; therefore folic acid deficiency as a limiting factor in hematopoiesis was shown not to exist in twenty-nine patients given adequate therapy with vitamin B_{12}.

3. The serum vitamin B_{12} level in twenty of thirty-five patients receiving 15 μg of vitamin B_{12} once every four weeks was less than 100 $\mu\mu$g per milliliter, which is characteristic of patients with pernicious anemia in relapse. It is greater than 100 $\mu\mu$g per milliliter in most patients receiving 30 μg of vitamin B_{12} once every four weeks. Inasmuch as the serum vitamin B_{12} concentration probably reflects tissue saturation, treatment with the larger dose of vitamin B_{12} appears to be more nearly adequate.

4. Administration of folic acid adversely affected the serum vitamin B_{12} level of eighteen of twenty-seven patients receiving 20 or 30 μg of vitamin B_{12} intramuscularly every two weeks, possibly by accelerating the utilization of vitamin B_{12}. Therefore, supplementation of inadequate intramuscular vitamin B_{12} therapy with folic acid, in addition to being unnecessary for nearly all patients, may actually be inadvisable unless larger doses of vitamin B_{12} are given.

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REFERENCES

1. Diez Rivas, F., Morales, F. H., and Meyer, L. H.: The Oral Use of Combined Vitamin B_{12} and Folic Acid in Tropical Sprue, Ann. Int. Med. **36**: 1076-1085, 1952.
2. Ross, J. F., Belding, H., and Paegel, B. L.: The Development and Progression of Subacute Combined Degeneration of the Spinal Cord in Patients With Pernicious Anemia Treated With Synthetic Pteroylglutamic (Folic) Acid, Blood **3**: 68-90, 1948.
3. Spies, T. D., Garcia Lopez, G., Milanes, F., Lopez Toca, R., and Reboredo, A.: Folic Acid and Vitamin B_{12} in Anemia, Gen. Practitioner **11**: 37, 1950.
4. Ungley, C. C.: Vitamin B_{12} and Other Dietary Factors in Megaloblastic Degeneration of the Cord, Proc. Roy. Soc. Med. **43**: 537-541, 1950.
5. Heinle, R. W., and Welch, A. D.: Folic Acid in Pernicious Anemia, J. A. M. A. **133**: 739-741, 1947.
6. Vilter, C. F., Vilter, R. W., and Spies, T. D.: The Treatment of Pernicious and Related Anemias With Synthetic Folic Acid. I. Observations on the Maintenance of a Normal Hematologic Status and on the Occurrence of Combined System Disease at the End of One Year, J. Lab. & Clin. Med. **32**: 262-273, 1947.
7. Harvey, E. A., Howard, I., and Murphy, W. P.: Absence of a Toxic Effect of Folic Acid on the Central Nervous System of Persons Without Pernicious Anemia, New England J. Med. **242**: 446-447, 1950.

8. Weissberg, J., McGavack, T. N., Vogel, M., and Keningsberg, S.: The Effect of Folic Acid on the Central Nervous System of Normal Subjects With Anemias Other Than Pernicious Anemia, Blood **5:** 148-154, 1950.
9. Chodos, R. B., and Ross, J. F.: The Effects of Combined Folic Acid and Liver Extract Therapy, Blood **6:** 1213-1233, 1951.
10. Conley, C. L., Green, T. W., Hartman, R. C., and Krevans, J. R.: Prolonged Treatment of Pernicious Anemia With Vitamin B_{12}, Am. J. Med. **13:** 284-293, 1952.
11. Evelyn, K. A.: A Stabilized Photoelectric Colorimeter With Light Filters, J. Biol. Chem. **115:** 63-75, 1936.
12. Wintrobe, M. M.: Clinical Hematology, Philadelphia, 1951, Lea & Febiger.
13. Lear, A. A., Harris, J. W., Castle, W. B., and Fleming, E. M.: The Serum Vitamin B_{12} Concentration in Pernicious Anemia, J. LAB. & CLIN. MED. **44:** 715-722, 1954.
14. Meacham, G. C., and Heinle, R. W.: Maintenance Therapy of Pernicious Anemia With Vitamin B_{12}, J. LAB. & CLIN. MED. **41:** 68-77, 1953.
15. Spray, G. H., and Witts, L. J.: The Utilization of Folic Acid Given by Mouth, Clin. Sc. **11:** 273-281, 1952.
16. Girdwood, R. H.: A Folic Acid Excretion Test in the Investigation of Intestinal Malabsorption, Lancet **2:** 53-60, 1953.
17. Ungley, C. C.: The Pathogenesis of Megaloblastic Anaemia and the Value of Vitamin B_{12}, Brit. J. Nutrition **6:** 299-315, 1952.
18. Ritz, N. D., Meyer, L. M., Brahin, C., and Sawitsky, A.: Further Observations on the Oral Treatment of Pernicious Anemia With Subminimal Doses of Folic Acid and Vitamin B_{12}, Acta Haematologica **5:** 334-338, 1951.
19. Reisner, E. H., Jr., and Weiner, L.: Studies on the Mutual Effect of Suboptimal Oral Doses of Vitamin B_{12} and Folic Acid in Pernicious Anemia, New England J. Med. **247:** 15-17, 1952.
20. Mollin, D. L., and Ross, G. I. M.: The Vitamin B_{12} Concentrations of Serum and Urine of Normals and of Patients With Megaloblastic Anaemias and Other Diseases, J. Clin. Path. **5:** 129-139, 1952.
21. Wallerstein, R. O., Schilling, R. S., Harris, J. W., Lear, A. A., and Castle, W. B.: Observations to be published.
22. Lear, A. A., Earle, C., Denny-Brown, D. E., and Castle, W. B.: Unpublished observations.
23. Spies, T. D., Vilter, R. W., and Ashe, W. F.: Pellagra, Beri Beri and Riboflavin Deficiencies in Human Beings, J. A. M. A. **113:** 931-937, 1939.
24. Davidson, L. S. P., and Girdwood, R. H.: The Imbalance of Vitamins With Particular Reference to Folic Acid, Lancet **1:** 360-363, 1948.

ERYTHROCYTE CHOLINESTERASE ACTIVITY IN NORMAL PREGNANCY AND IN MEGALOBLASTIC AND OTHER ANEMIAS OF PREGNANCY AND THE PUERPERIUM

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IT HAS been demonstrated repeatedly that erythrocyte cholinesterase activity (RBC ChE) in the presence of anemia may be quite different from normal.[1-4] During relapse in Addisonian pernicious anemia, it is usually low, but, following a hematopoietic response, RBC ChE becomes abnormally high and then returns to normal several weeks later. Experimental hemorrhage in animals is followed by an appreciable increase in RBC ChE. Erythrocytes obtained from such animals during recovery and from patients with pernicious anemia during response to therapy have been separated into reticulocyte-rich and reticulocyte-poor fractions by differential centrifugation, and the RBC ChE of each fraction measured. Much greater enzyme activity was present in the reticulocyte-rich fraction in each instance, indicating that young red cells have greater cholinesterase activity than do older erythrocytes.[3, 5]

These findings have resulted in the suggestion by Sabine[5] that the measurement of RBC ChE might be used as a test of bone marrow function in individuals with severe anemias. Scudamore and associates,[4] however, have pointed out that the paucity of data in cases of hemolytic anemia is a main weakness in this hypothesis.

The present investigation was carried out to study the effect of the increased erythrocyte production of normal pregnancy on RBC ChE, to compare RBC ChE in so-called pernicious anemia of pregnancy and the puerperium with red-cell enzyme activity in Addisonian pernicious anemia, and to study RBC ChE in various hemolytic states.

METHODS

Erythrocyte cholinesterase activity was determined by the method of Michel.[6] With the use of this technique, a measurement is made of the decrease in the pH of a balanced buffer solution due to the hydrolysis of acetylcholine by the RBC ChE in a measured volume of erythrocytes. The enzyme activity is expressed as the fall in pH per hour (Δ pH per hour). In the cases of megaloblastic anemia of pregnancy and the puerperium, plasma cholinesterase activity was determined also by the method of Michel. The red cells and plasma were collected as recommended, and, after mixing with an equal volume of normal saline solution, they were either studied immediately or were promptly frozen and stored until just before assay.

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RESULTS

Normal Pregnancy and the Puerperium.—The RBC ChE was determined in 18 normal nonpregnant women, in 81 normal women in various stages of gestation, in 16 normal women at the time of delivery and in the fetus (cord blood) from each of these deliveries, and in 17 normal women six weeks following delivery.

There was a slight but persistent increase in RBC ChE as pregnancy advanced. In the third trimester the mean RBC ChE for 36 women was 0.81 pH unit per hour compared with an average activity of 0.74 pH unit per hour in 18 normal nonpregnant women. This difference is highly significant ($P > 0.01$). The mean activity for 16 normal patients at the time of delivery was also 0.81 pH unit per hour, but in cord blood obtained at delivery it was only 0.50 pH unit per hour. The mean RBC ChE and standard deviation for each group are shown in Table I.

TABLE I. RBC ChE ACTIVITY IN NORMAL NONPREGNANT WOMEN, IN NORMAL WOMEN IN VARIOUS STAGES OF GESTATION AND SIX WEEKS POST PARTUM, AND IN NORMAL TERM FETUSES

	NUMBER	MEAN RBC ChE IN pH UNITS PER HOUR
Nonpregnant	18	0.74 S.D. ± 0.07
First trimester	14	0.76 S.D. ± 0.07
Second trimester	31	0.79 S.D. ± 0.07
Third trimester	36	0.81 S.D. ± 0.09
Delivery	16	0.81 S.D. ± 0.10
Fetus	16	0.50 S.D. ± 0.04
Post partum	17	0.78 S.D. ± 0.10

Megaloblastic Anemia of Pregnancy and the Puerperium.—RBC and plasma ChE were measured before, during, and after treatment in 3 cases of megaloblastic anemia of pregnancy and the puerperium. Each case is summarized following.

CASE 1.—G. M., a 24-year-old Negro woman, was first seen on June 17, 1953, when she was seventeen weeks' pregnant. At this time the hematocrit was 25, there was no history of blood loss, and the erythrocytes did not sickle in a solution of sodium metabisulfite. Therapy with oral iron was started.

During the next three months there was persistent anorexia. Since early in the pregnancy, meat and leafy vegetables were eaten only rarely; the diet consisted almost entirely of potatoes, bread, orange juice, grape juice, green beans, and "Kool Ade." By September 15 the patient had noted marked weakness, shortness of breath on exertion, frequent loose bowel movements, and considerable weight loss. At this time she was admitted to the hospital where laboratory studies revealed the following: hemoglobin 4.80 Gm., red blood cell count 1.98 million, hematocrit 16, white blood count 6.5 thousand, reticulocytes 1.2 per cent, platelets 75,000, total serum proteins 6.2 Gm., serum bilirubin 0.2 mg. direct and 1.1 mg. total, free acid in the gastric juice after histamine, and no blood in the feces. The erythrocytes appeared hypochromic and showed considerable variation both in size and shape; the platelets were reduced in number. The bone marrow contained numerous megaloblasts.

Five hundred milliliters of whole blood was given, followed during the next six days by 1,200 mg. of saccharated iron oxide injected intravenously. Vitamin B_{12} 1 μg and ascorbic acid 50 mg. intramuscularly daily were started four days after the last injection of iron. This

therapy was continued for six days; at the end of this time the bone marrow was still megaloblastic and there was no evidence of hematological or symptomatic improvement. During this period the RBC ChE ranged from 0.57 to 0.61 pH unit per hour, the reticulocyte level remained at about 1 per cent, and the hematocrit was 18 to 19.

On October 1, sixteen days after admission, therapy with Leucovorin, 3 mg. intramuscularly each day, was started. Two days later the reticulocyte count was 4.2 per cent, and four days after the first injection of Leucovorin it reached 19.9 per cent. Two days following the onset of the reticulocytosis, the RBC ChE started to rise and continued to increase to reach 1.1 pH units per hour on November 6. By this time the hematocrit had risen to 38. The RBC ChE then slowly fell during the next six weeks to 0.78 pH unit per hour.

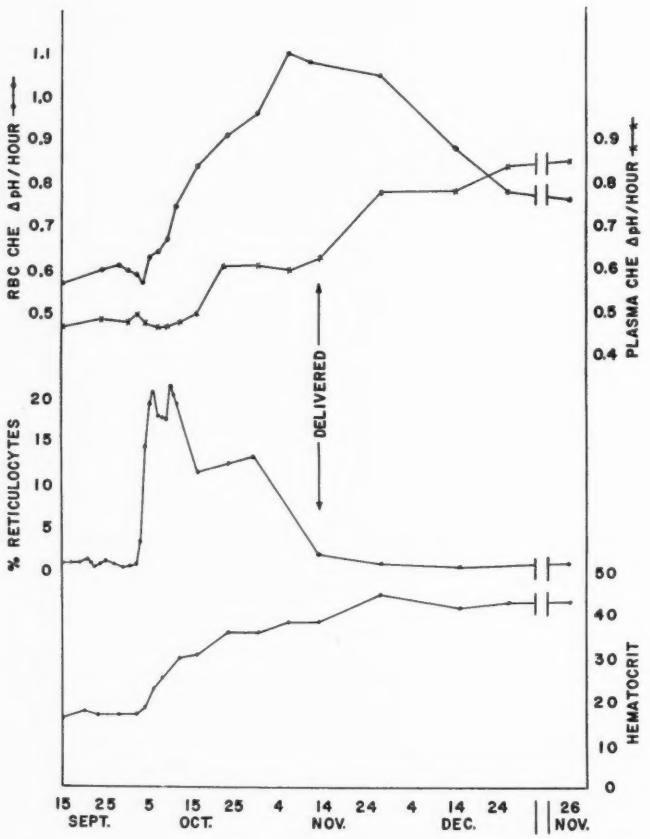

Fig. 1.—Case 1, G. M., megaloblastic anemia during pregnancy.

During the first month after admission, the plasma ChE remained at a level of 0.47 to 0.50 pH unit per hour. Two weeks after the start of therapy with Leucovorin, it rose to 0.60 pH unit per hour where it remained until delivery on November 13. Following delivery the plasma activity progressively rose to reach 0.84 pH unit per hour on December 26. Eleven months later it was 0.85 pH unit per hour. At this time the patient was asymptomatic and hematologically normal. The results are summarized in Fig. 1.

Case 2.—I. G., an 18-year-old Negro woman known to have sickle-cell anemia, was admitted to the hospital on February 27, 1953, with diagnoses of severe anemia due to sickle-cell

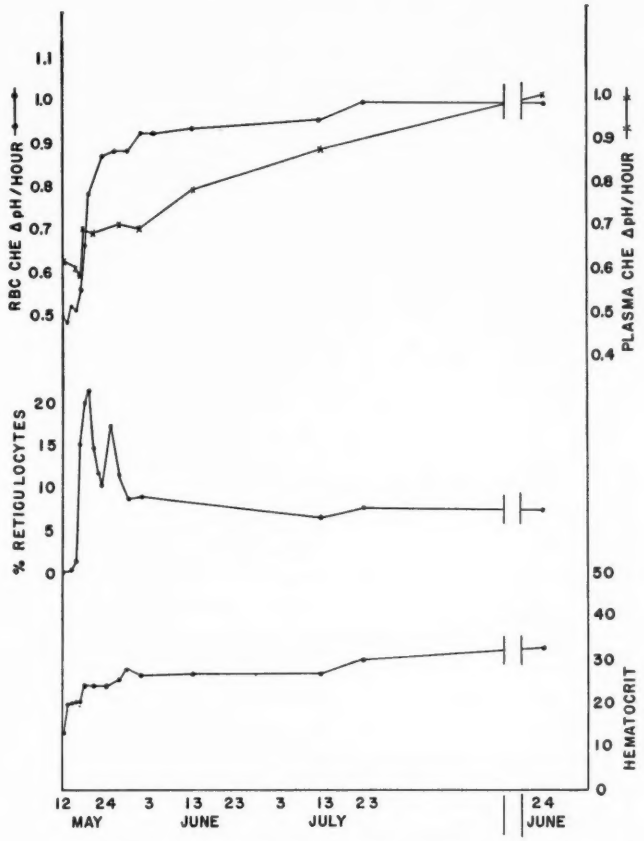

Fig. 2*A*.—Case 2, I. G., megaloblastic anemia during pregnancy associated with sickle-cell anemia.

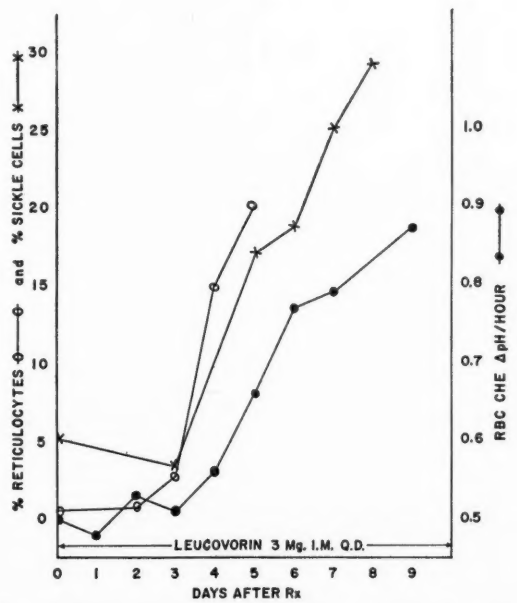

Fig. 2*B*.—Case 2, I. G. Plotted are the percentage of sickle cells and reticulocytes and the rise in RBC ChE following therapy with Leucovorin.

anemia, acute pyelonephritis, and pregnancy of about thirty weeks' duration. During the next two and one-half months there were repeated episodes of pyelonephritis and superficial thrombophlebitis. Penicillin, chlortetracycline, streptomycin, and Gantrisin were administered in various combinations. Marked anorexia persisted throughout this period. The hematocrit continued to fall, reaching as low as 12 in spite of transfusions with a total of 15 pints of whole blood which were given in an attempt to keep the hematocrit above 15. On May 11, only 5.6 per cent of the red cells of the peripheral blood sickled in sodium metabisulfite solution. The following day the bone marrow was found to contain numerous megaloblasts. At this time the hematocrit was 13, red cell count 1.6 million, hemoglobin 4.3 Gm., reticulocytes 0.1 per cent, white blood cell count 5.1 thousand, and platelet count 76,000. Therapy with Leucovorin, 3 mg. a day intramuscularly, was started. That evening the patient spontaneously went into labor and delivered a sluggish term-sized infant. Because of the low hematocrit and moderately excessive blood loss during the immediate puerperium, 2 more units of blood was given.

Three days after the start of Leucovorin therapy the reticulocyte count rose to 3.2 per cent and the fourth day it was 15.1 per cent. Concomitant with the initial reticulocytosis there was an almost identical increase in the number of circulating sickle cells.

Prior to the reticulocyte response, the RBC ChE was about 0.50 pH unit per hour. Within twenty-four hours after the initial increase in reticulocytes, the RBC ChE started to rise. For the next several days the rates of increase in the concentration of reticulocytes, sickle cells, and RBC ChE were nearly parallel. The increase in enzyme activity continued, at first rapidly and then more slowly, until it reached 0.95 pH unit per hour about three weeks after the start of therapy. Thirteen months later the RBC ChE was still at this level and was accompanied by an elevated reticulocyte count.

The plasma ChE rose, initially somewhat rapidly and then much more slowly, from a pretreatment level of about 0.62 pH unit per hour to 0.99 pH unit per hour two and one-half months later. The results are summarized in Figs. 2*A* and 2*B*.

CASE 3.—D. C., a 19-year-old Negro woman, was admitted with severe anemia accompanied by diarrhea, anorexia, and fever seven weeks following delivery.

Laboratory studies revealed the following: red blood count 1.80 million, hemoglobin 5.9 Gm., hematocrit 16, white blood count 4.6 thousand, reticulocytes 0.8 per cent, and platelets 38,000. Aspirated bone marrow contained numerous megaloblasts. Achlorhydria was present even after the injection of histamine, but two weeks later free acid was found.

Five hundred milliliters of whole blood was given, and 5 μg of vitamin B_{12} and 500 mg. of sodium ascorbate were injected intramuscularly each day. Prior to any reticulocyte response, the RBC ChE averaged 0.66 pH unit per hour. On the sixth day of therapy, the reticulocyte count started to increase. This was followed within forty-eight hours by an increase in the RBC ChE. The erythrocyte enzyme activity continued to rise to reach a plateau three weeks later of 1.0 pH unit per hour. By this time the hematocrit had risen to 38. During the next two months there was a gradual decrease in enzyme activity to 0.85 pH unit per hour. Eleven months later the RBC ChE remained at this level and the hematocrit was 41.

Prior to therapy with vitamin B_{12} and ascorbic acid and for about two weeks thereafter, the plasma ChE activity ranged from 0.52 to 0.58 pH unit per hour. During the next four weeks it progressively rose to reach 0.79 pH unit per hour. These data are shown in Fig. 3.

Cases of Increased Erythrocyte Destruction or Acute Hemorrhage.—RBC ChE, reticulocyte counts, and hematocrits were determined one or more times in 2 cases of sickle-cell anemia, in one case of severe anemia probably due to sickle-thalassemia combination, in 3 cases of anemia associated with the combination of sickle hemoglobin and hemoglobin C, in 3 cases of eclampsia during an apparent

hemolytic episode, and in one case of anemia following an acute massive hemorrhage from the uterus. The various types of hemoglobins were demonstrated by means of filter paper electrophoresis. The results are summarized in Table II.

In one case of sickle-C disease (F. B.), the RBC ChE was 0.96 and 1.02 pH units per hour while the reticulocyte counts were 17.7 and 23 per cent. Eleven months later the RBC ChE had dropped to 0.77 pH unit per hour and the reticulocyte count was then only 2.7 per cent. In the case of possible sickle-thalassemia disease, when the reticulocyte level was elevated, the RBC ChE was higher than when the reticulocyte count was low, but at no time did the RBC ChE exceed the range for normal nonpregnant individuals (0.74 pH unit per hour, S.D., ± 0.07).

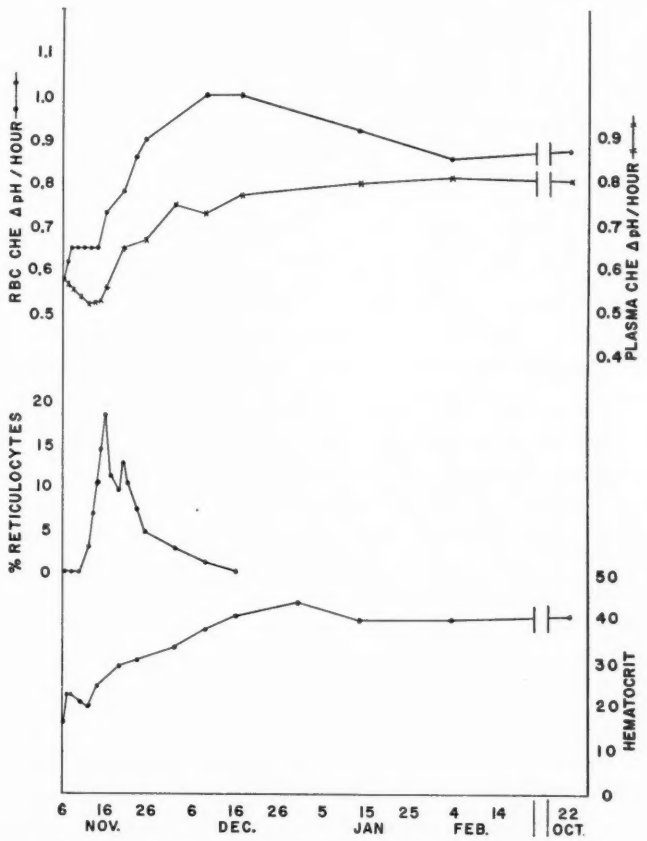

Fig. 3.—Case 3, D. C., megaloblastic anemia during the puerperium.

In 7 of the 10 cases of increased erythropoietic activity due to either increased red cell destruction or acute blood loss, there was no consistent relationship between the level of RBC ChE and the number of circulating reticulocytes. In one case of sickle-cell anemia (M. C.), the RBC ChE on occasion was abnormally high, but at other times it was normal in spite of a large number of circulating reticulocytes. In the 3 cases of eclampsia, the RBC ChE was normal although the reticulocyte level was as high as 9 per cent.

TABLE II. RBC ChE, RETICULOCYTE COUNTS, AND HEMATOCRITS IN PROBABLE SICKLE-THALASSEMIA ANEMIA, SICKLE-CELL ANEMIA, SICKLE-HEMOGLOBIN C DISEASE, INTRAVASCULAR HEMOLYSIS OF ECLAMPSIA, AND SEVERE ANEMIA DUE TO ACUTE HEMORRHAGE

DIAGNOSIS	PATIENT	DATE	RBC ChE pH/HOUR	RETICULOCYTES (PER CENT)	HEMATOCRIT
Sickle-thalassemia anemia	E. G.	Oct. 8	0.70	7.7	26
		Oct. 14	0.73	13.4	25
		Jan. 6	0.59	1.7	39
		July 13	0.61	0.6	40
Sickle-cell anemia	M. C.	Sept. 30	0.78	17.0	28
		Oct. 20	0.90	16.5	27
		Oct. 26	0.98	11.9	23
		Dec. 22	0.71	9.0	29
		Dec. 29	0.73	10.5	29
	I. G.	June 24	0.98	7.7	33
Sickle-C anemia	F. B.	Aug. 11	0.96	17.7	23
		Aug. 12	1.02	23.0	22
		July 14	0.76	2.7	32
	B. J.	Sept. 22	0.77	1.6	33
	B. C.	Apr. 14	0.76	2.5	30
		July 5	0.85	2.8	33
Eclampsia	M. S.	May 31	0.80	7.7	39
		June 8	0.77	6.4	40
	P. H.	Dec. 17	0.68	5.1	37
		Dec. 21	0.72	6.0	36
	A. C.	Nov. 24	0.66	6.2	35
		Nov. 30	0.66	9.1	34
		Dec. 4	0.64	5.6	36
Acute hemorrhage	M. A.	Oct. 6	0.90	4.5	20

DISCUSSION

A slight but significant increase in RBC ChE per unit volume of red cells occurs during pregnancy. Since mean corpuscular volumes were not calculated for each specimen of blood obtained from the normal pregnant and postpartum patients, it cannot be said with certainty that the increase in RBC ChE represents an actual increase in mean cell esterase activity; however, mean corpuscular volumes were determined in 16 specimens of blood from the mothers at delivery and the average was 89 millimicrons.

The usual increase in plasma volume during pregnancy probably is about 25 to 35 per cent;[8, 9] therefore, in the nonanemic pregnant patients, the rate of erythrocyte production must have exceeded erythrocyte destruction and, thus, a greater proportion of the circulating red cells must have been released more recently from the bone marrow than was true in blood from the normal nonpregnant individuals. The alternative is that the lifespan of circulating erythrocytes during pregnancy must be significantly lengthened. The results of measurements of red cell survival during pregnancy do not support this possibility.[10] Since studies by Pritchard[3] and by Sabine[5] indicate that young

erythrocytes obtained following hemorrhage or during the reticulocytosis following treatment in cases of pernicious anemia contain significantly more cholinesterase activity than do the older circulating red cells, it would seem most likely that the progressive increase in erythrocyte cholinesterase activity during pregnancy results from an increase in the concentration of younger red cells circulating in the blood.

Per unit volume of red cells, there is a marked difference between RBC ChE in red cells from either nonpregnant or pregnant normal adults and RBC ChE in erythrocytes from normal term fetuses. When RBC ChE is expressed as mean cell esterase activity, however, this difference is not as marked, since the mean corpuscular volumes of the fetal red cells averaged 112 millimicrons compared with 89 millimicrons for the red cells from the normal mothers. Therefore, while maternal RBC ChE per unit volume was 62 per cent greater than fetal RBC ChE, mean red-cell esterase activity in the mother was only 30 per cent greater than mean red-cell esterase in the fetus.

Both the increase in maternal RBC ChE as pregnancy advanced and the lower RBC ChE in the fetus than in the mother differ from the changes found in plasma ChE during pregnancy.[7] Plasma ChE progressively decreases as pregnancy advances from the nonpregnant level of 0.84 pH unit per hour to 0.66 pH unit per hour during the third trimester. The maternal mean plasma ChE and the plasma ChE in the term fetus are identical at the time of delivery.

During the period prior to a hematopoietic response in the 3 cases of megaloblastic anemia of pregnancy and the puerperium, the RBC ChE was reduced. This reduction held true whether the activity was calculated per cell or per unit volume of red cells, since in all 3 cases the erythrocytes were normocytic. Within twenty-four to seventy-two hours after the first detectable increase in the reticulocyte count, the RBC ChE started to increase. As erythropoiesis continued, with the result that most of the circulating erythrocytes were young cells, the RBC ChE rose above the normal range. Then, in Cases 1 and 3, the activity slowly dropped to normal and remained there. The changes in RBC ChE following therapy in these 2 cases were very similar to those which have been observed previously in Addisonian pernicious anemia.[1, 2, 4]

In Case 2, however, in which megaloblastic anemia was associated with sickle-cell anemia, the initial rise in RBC ChE was not followed by a fall to the normal range; instead, it was still elevated thirteen months later. This difference in the ultimate level of RBC ChE between Case 2, with associated sickle-cell anemia, and Cases 1 and 3 is consistent with the concept that an elevated RBC ChE results from an increase in the number of circulating young erythrocytes, since ordinarily in sickle-cell anemia there is an increased rate of red cell production and many young cells in the circulation.

The increase in plasma ChE which followed therapy in these 3 cases was similar to the rise noted in true pernicious anemia after a hematological response,[1, 2] but any evaluation of the changes in plasma ChE during and after remission in Cases 1 and 2 is difficult, since in normal pregnancy during the

third trimester there is, on the average, a 21 per cent decrease in plasma ChE. Within six weeks following delivery, however, the plasma ChE has returned to the prepregnant level in normal patients,[7] so that it would seem probable that at least in Case 3 the increase in plasma ChE was related to the remission produced by vitamin B_{12} and ascorbic acid since pretreatment plasma ChE measurements were first carried out seven weeks after delivery.

In spite of the evidence set forth in previous reports[3, 5] that younger erythrocytes usually possess greater cholinesterase activity than do older red cells, the present study of patients with increased erythroid activity due to a hemolytic process or following acute hemorrhage indicates that a measure of RBC ChE more often than not is of no value as a means of detecting such bone marrow hyperactivity. In only 3 of 10 cases would the RBC ChE suggest an increased rate of red cell production, and, in one of these 3 cases (M. C., sickle-cell anemia), repeated measurements over a three-month period did not consistently result in the detection of an elevated RBC ChE although there was an appreciable reticulocytosis at the time of each study.

This lack of consistent correlation between RBC ChE and the degree of reticulocytosis in hemolytic anemias is not peculiar to this enzyme alone. Even though reticulocytes have significantly greater glycolytic activity than do older red cells, Hollingsworth[11] did not find a consistently good correlation between red-cell glycolytic activity and the degree of reticulocytosis in cases of hemolytic anemias.

SUMMARY

There is a slight but significant increase in RBC ChE during normal pregnancy. RBC ChE in the normal term fetus is lower than in the mother.

Prior to remission in megaloblastic anemia of pregnancy and the puerperium, RBC ChE is low; following a hematological response RBC ChE becomes appreciably elevated and then ultimately falls to the normal range in otherwise uncomplicated cases.

Although previous studies strongly suggest that RBC ChE is greater in young red cells, RBC ChE in cases of hemolytic anemias frequently is not significantly elevated in spite of evidence of bone marrow hyperactivity.

The measurement of RBC ChE is not a satisfactory test to detect the presence of bone marrow erythroid hyperactivity.

REFERENCES

1. Sabine, J. C.: Cholinesterase of Blood Cells and Plasma in Blood Dyscrasias, With Special Reference to Pernicious Anemia, J. Clin. Invest. **19**: 833, 1940.
2. Meyer, L. M., Sawitsky, A., Ritz, N. D., and Fitch, H. M.: A Study of Cholinesterase Activity of the Blood of Patients With Pernicious Anemia, J. Lab. & Clin. Med. **33**: 189, 1948.
3. Pritchard, J. A.: Erythrocyte Age and Cholinesterase Activity, Am. J. Physiol. **158**: 72, 1949.
4. Scudamore, H. H., Vorhaus, L. J., and Kark, R. M.: Observations on Erythrocyte and Plasma Cholinesterase Activity in Dyscrasias in the Blood, Blood **6**: 1260, 1951.

5. Sabine, J. C.: The Cholinesterase of Erythrocytes in Anemias, Blood **6**: 151, 1951.
6. Michel, H. O.: An Electrometric Method for the Determination of Red Blood Cell and Plasma Cholinesterase Activity, J. LAB. & CLIN. MED. **34**: 1564, 1949.
7. Pritchard, J. A.: Plasma Cholinesterase Activity in Normal Pregnancy and in Eclamptogenic Toxemias, Am. J. Obst. & Gynec. In press.
8. Deickmann, W. J., and Wegner, C. J.: The Blood in Normal Pregnancy. I. Blood and Plasma Volumes, Arch. Int. Med. **53**: 71, 1934.
9. White, R.: Symposium on Haemodynamics in Pregnancy. III. Blood Volume in Pregnancy **57**: 14, 1950.
10. Lowenstein, L., and Edington, R.: Red Cell Survival in Normal Pregnancy and in Toxemia of Pregnancy, Presented at Fortieth Clin. Congress, Am. Coll. Surgeons, Nov. 16, 1954, Atlantic City, N. J.
11. Hollingsworth, J. W.: Erythrocyte Glycolysis in Hemolytic Disease, J. LAB. & CLIN. MED. **45**: 920, 1955.

SERUM TRANSAMINASE ACTIVITY

Observations in a Large Group of Patients

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GLUTAMIC oxalacetic transaminase, henceforth referred to as transaminase, is present in high concentration in such tissues as heart, liver, kidney, muscle and brain.[1] This enzyme catalyzes the following reversible reaction:

$$\text{glutamate} + \text{oxalacetate} \xrightleftharpoons[\text{glutamic-oxalacetic transaminase}]{} \text{aspartate} + \text{alpha-ketoglutarate}$$

in which the alpha amino nitrogen of the amino acid is transferred for the alpha-keto group of the alpha-keto acid with the synthesis of a second amino acid and a second alpha-keto acid. Recent studies have demonstrated the presence of this enzyme in all normal human sera studied[2] and its significant elevation in diseases associated with myocardial[3] and hepatic necrosis.[4] Experimentally, infarctions produced by ligation of the coronary, pulmonary, renal, splenic, and mesenteric vessels produced significant elevations of the serum transaminase activity, which were roughly correlated with the degree of tissue involvement.[5] Significant elevations of transaminase activity were not observed with temporary occlusion of these same vessels.[5] Experimental hepatic necrosis produced by carbon tetrachloride has resulted in elevations of serum transaminase activity of 50 to 1,000 times normal.[4]

It is the purpose of this report to describe the levels of serum transaminase activity in a group of 400 patients with a variety of diseases, and to discuss the use of this procedure as a diagnostic method.

METHODS

Serum transaminase activity was assayed by the method of Karmen.[2] Wherever possible, serial specimens were obtained for assay. The whole blood specimens were kept under refrigeration until separation of the serum and assay. No significant decrease in transaminase activity was noted during forty-eight-hour periods of refrigeration. Duplicate samples were consistently reproducible within 10 units per milliliter of serum.

Transaminase activity of bile and urine was determined by the method of Karmen,[2] modified only in that the solution to be assayed was substituted for serum.

Preliminary observations, pertinent to the study, included:

A. *Normals.*—Fifty assays of the serum transaminase of 15 young healthy adults ranged from 7 to 40 units per milliliter of serum, with an average of 20 ± 8 units per milliliter. An arbitrary definition of ranges of activity was made as follows:

Normal	—	Up to 40 units per milliliter of serum.
Border line	—	41 to 50 units per milliliter of serum.
High	—	Above 50 units per milliliter of serum.

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Multiple transaminase assays on these 15 normals did not vary by more than 10 units per milliliter of serum and were independent of the time of day, relation to meals, and previous exercise.*

B. *Other Body Fluids.*—In order to gain information about modes of excretion of transaminase, urine and bile were studied for their content of the enzyme. Insignificant amounts of transaminase were present in normal urine. In patients with high serum transaminase levels, significant amounts of enzyme activity appeared in the urine, but the urinary levels were very low in comparison to the serum levels. Fresh bile was obtained from two patients at laparotomy, one with a normal biliary tree, the other with chronic cholecystitis and lithiasis but without common duct obstruction. Both specimens of bile contained 2,000 units of transaminase activity per milliliter of bile, respectively, indicating the presence of large amounts of transaminase in the bile.

RESULTS

Table I summarizes the results obtained in the 400 cases studied. The cases have been divided into major disease categories. Under each category are shown the total studied, as well as the number of cases with normal, borderline, and high transaminase levels. Although multiple or serial determinations were obtained in most instances, the highest transaminase activity observed was used in constructing the table.

TABLE I. SUMMARY OF THE SERUM TRANSAMINASE ACTIVITIES OF THE 400 CASES STUDIED

DIAGNOSIS	TOTAL NUMBER OF CASES	TRANSAMINASE LEVELS		
		NORMAL	BORDERLINE	HIGH
Acute myocardial infarction	117	4	5	108
a. ECG diagnostic	84	4	1	79
b. ECG not diagnostic	33	0	4	29
A. Autopsy evidence	8	0	1	7
B. Clinical evidence	25	0	3	22
Anginal syndrome	69	64	5	0
Acute pulmonary edema	16	12	3	1
Chronic congestive cardiac failure	11	11	0	0
Benign idiopathic pericarditis	5	5	0	0
Cardiac arrhythmias	15	7	0	8
Cerebral vascular accidents	6	5	0	1
Hepatitis	15	1	0	14
I. Infectious mononucleosis	6	0	0	6
II. Infectious or serum hepatitis	8	0	0	8
III. Chronic granuloma of liver	1	1	0	0
Cirrhosis of the liver	13	5	0	8
Obstructive jaundice	10	2	0	8
Carcinomatosis	10	5	0	5
Pancreatitis	16	2	3	11
Miscellaneous	97	88	2	7

Acute Myocardial Infarction.—There were 117 acute myocardial infarctions occurring in 107 patients. The electrocardiogram revealed the typical pattern of acute myocardial infarction in 84 instances. Fig. 1 cites the results of serial transaminase determinations in these 84 infarcts. It will be noted that the serum transaminase level became significantly elevated approximately six hours after the onset of chest pain, rising to a peak at about twenty-four hours after the onset of pain. The levels then returned toward normal, reaching the

*Eight of our normal group participated in a strenuous basketball game of three hours' duration, during which they maintained a persistent tachycardia. No significant elevation of their serum transaminase occurred as a result of this activity.

normal range on the third to the sixth day. The serial changes in serum transaminase activity of two patients of this group have been charted in Fig. 1, to demonstrate that the duration of elevation of the serum transaminase activity appeared to be related to the height of activity achieved at the twenty-four hour peak.

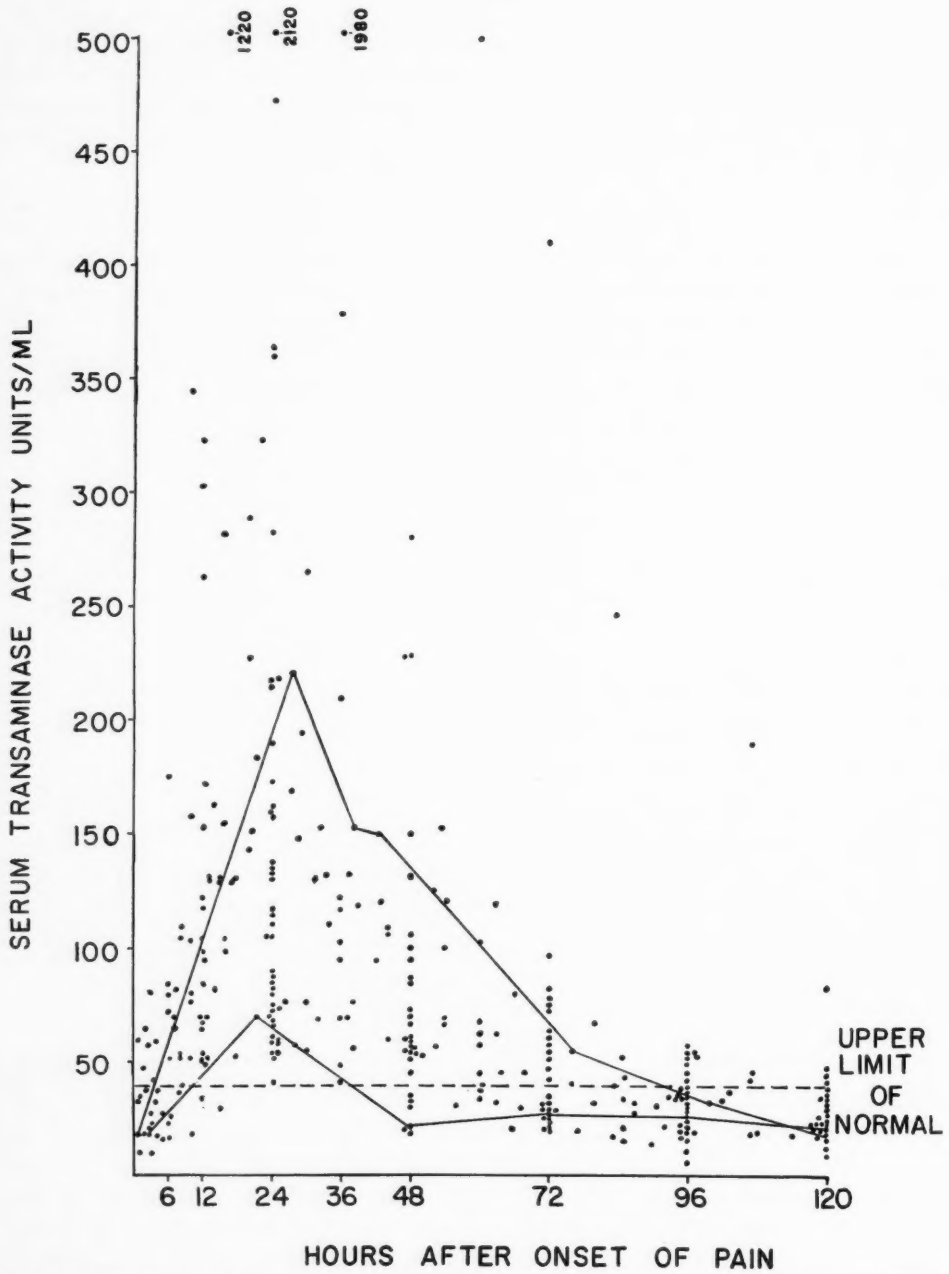

Fig. 1.—Serum transaminase levels observed after the onset of pain in 84 instances of proved myocardial infarction.

Of these 84 patients, 79 developed abnormally high serum transaminase values, 1 had a value in the borderline range, and 4 were in the normal range. In the latter group, one patient died one and one-half hours after the onset of pain; one had her acute episode seven days prior to the initial sampling; one had no specimens drawn between the sixth to the sixtieth hour; and one had his acute episode three days prior to the first assay. The patient with the value in the borderline range only developed a level of 41 units, but this proved to be 3½ times his subsequent levels.

An additional 33 cases were diagnosed as having had myocardial infarcts without electrocardiographic confirmation. Eight were proved to have acute infarcts at autopsy, whereas the diagnosis in the other 25 cases was made on clinical evidence. In almost all instances the electrocardiogram was abnormal but not diagnostic of infarction. In all instances of the group with infarcts at autopsy, except for one patient who died one and one-half hours after the onset of pain, the serum transaminase was in the high range. Twenty-two of the 25 patients in whom infarction was diagnosed on clinical grounds had a high serum transaminase level. The results were border line in the other 3, but specimens were not obtained at optimal times after the onset of pain.

Although there were insufficient data to correlate serum transaminase activity with size of infarct or prognosis, it was observed that those patients who had very high serum transaminase levels (i.e., above 200 units) were usually desperately ill and had a high mortality rate. Of 111 patients who survived the first day of their insult, 25 developed serum transaminase activities of greater than 200 units. Thirteen of these patients died (52 per cent mortality), and an additional 7 were in severe shock for protracted periods of time. In contrast, 14 of the 86 patients who did not reach levels of 200 died (16 per cent mortality), and the incidence of shock was not as great.

Comment: Of the entire group of 117 diagnosed infarctions, 108, or 92 per cent, were associated with an abnormally high serum transaminase. When specimens were taken at proper intervals, only one case of the entire group failed to develop a high value. The optimal time for observing the peak transaminase activity was twenty-four hours after the onset of pain. Evidence was obtained which suggests that the appearance of very high transaminase levels may be a poor prognostic sign.

Anginal Syndrome.—Of 69 cases with the anginal syndrome, 64 had normal serum transaminase activity and 5 reached borderline levels. In no instance was a high level observed. Twenty-one of the patients with the anginal syndrome had only a single determination of transaminase activity, specimens being obtained twenty-four to fifty-two hours after the onset of pain. The remaining 48 cases had serial determinations, with 36 exhibiting no significant changes. Twelve cases, all with abnormal electrocardiograms, did show significant serial changes in transaminase (greater than 20 units) even though the maximal value did not exceed the normal or borderline range. An example of this latter type of response is shown in Fig. 2.

Comment: The serum transaminase remains in the normal or near normal range with the anginal syndrome. Of interest is that some of these patients developed serial changes in transaminase activity which might, in the light of experimental work,[5] suggest the presence of small or subclinical infarcts.

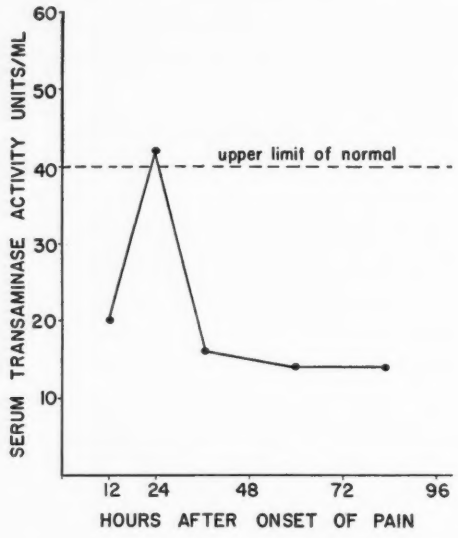

Fig. 2.—Significant serial changes in serum transaminase activity observed in a patient with the anginal syndrome. The maximum level reached is just at the upper limit of the normal range.

Acute Pulmonary Edema.—Of 16 patients with acute pulmonary edema in whom acute myocardial infarction was not the precipitating cause, the serum transaminase activity was normal in 12, border line in 3, and elevated in 1. The latter case was complicated by the presence of a rapid auricular fibrillation (180 per minute). The association of a high transaminase with rapid arrhythmias will be subsequently discussed.

Chronic Congestive Cardiac Failure.—There were 11 cases with chronic congestive cardiac failure due to arteriosclerotic heart disease. All of these patients had normal serum transaminase levels. The level of serum transaminase was not altered by treatment for the cardiac failure or by the presence of digitalis intoxication.

Benign Idiopathic Pericarditis.—Normal serum transaminase activity was observed in all 5 cases of benign idiopathic pericarditis studied.

Cardiac Arrhythmias.—Serum transaminase levels were studied in 15 instances of cardiac arrhythmias, occurring in 14 patients. The results are shown in Table II. The patients have been divided into two groups according to their ventricular rates. The 7 patients with ventricular rates of 120 to 160 beats per minute had normal serum transaminase activity, while the 8 instances of very rapid arrhythmias were associated with high transaminase levels. It is to be noted, however, that 2 of the patients (E. H. and S. B.) with normal transaminase levels had previously had very rapid rates. Patient O. P., with a rapid ventricu-

lar tachycardia, developed a transaminase level of 750 units. Post-mortem examination revealed extensive centrilobular liver cell necrosis but no myocardial infarct or myocarditis.

Comment: The mechanism for the elevation of serum transaminase in some of the patients with cardiac arrhythmias is obscure. Perhaps centrilobular liver cell necrosis may contribute to this level, as suggested by the one patient who came to autopsy. In normals, tachycardia produced by strenuous exercise did not elevate the serum transaminase level.

TABLE II. SERUM TRANSAMINASE LEVELS IN PATIENTS WITH CARDIAC ARRHYTHMIAS

CASE	AGE (YR.)	SEX	TYPE OF ARRHYTHMIA	VENTRICULAR RATE	SERUM TRANSAMINASE UNITS/ML.	REMARKS
			A. Ventricular Rate 120 to 160 Per Minute			
M. N.	67	M	Paroxysmal auricular tachycardia	150	32	
E. H.	52	F	Paroxysmal auricular tachycardia	140	31	Two hours previously had rate of 250 to 300
M. H.	40	F	Paroxysmal auricular tachycardia	150	15	
S. B.	26	M	Auricular flutter	120-140	14	Had rate of 150 to 200 the previous day
L. B.	47	M	Nodal tachycardia	140-160	23	
E. S.	66	F	Auricular fibrillation	135	29	
N. F.	68	M	Flutter-fibrillation	130-140	38	
			B. Ventricular Rate 180 to 300 Per Minute			
J. W.	55	M	Ventricular tachycardia	200-250	79	
J. W.	55	M	Ventricular tachycardia	250	56	
A. W.	71	M	Auricular flutter	200	143	
B. R.	61	F	Flutter-fibrillation	180-200	122	
O. P.	49	M	Ventricular tachycardia	200-250	750	At post-mortem, exhibited centrilobular liver cell necrosis
L. M.	66	M	Auricular flutter	200-250	67	
M. K.	60	M	Auricular fibrillation	180-200	110	
S. F.	75	F	Flutter-fibrillation	190	112	

Cerebral Vascular Accidents.—Four of the 5 patients with cerebral vascular accidents had normal serum transaminase levels. The patient with a high level (570 units) entered in profound shock and at autopsy exhibited a massive area of encephalomalacia. There were no significant abnormalities in the heart and liver.

Hepatitis.—The serum transaminase was high in 14 of the 15 patients with hepatitis. The only normal value was observed in a nonjaundiced patient who, on liver biopsy (at exploratory laparotomy), proved to have a chronic granulomatous lesion of the liver.

All 6 patients with hepatitis due to infectious mononucleosis (2 were not icteric) had high serum transaminase activities ranging from 50 units to 150

units. The 8 patients with infectious or serum hepatitis had very high transaminase levels ranging from 173 units to 2,200 units. Seven of these latter patients had levels above 400 units.

Fig. 3 describes serial transaminase determinations in an uncomplicated case of infectious hepatitis. Note the persistence of very high serum transaminase levels for approximately one week, followed by a slow approach toward the normal range.

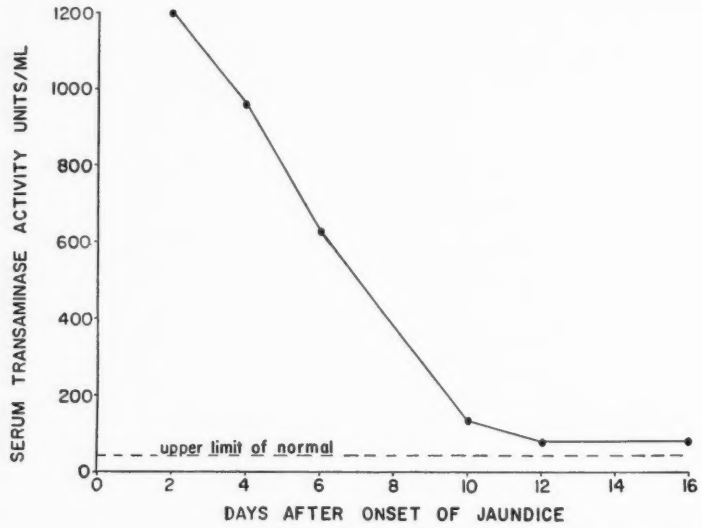

Fig. 3.—Serial serum transaminase levels in a patient with acute infectious hepatitis. Sixteen days after the onset of jaundice, the level is still significantly elevated.

Comment: Experimentally, hepatic necrosis has been associated with marked serum transaminase rises which have remained high for variable periods and fallen with or before clinical improvement.[4] Our observations in patients with hepatitis are in accord with these experimental studies. At present, sufficient data are not available to correlate the serum transaminase level with other tests for hepatic function.

Cirrhosis of the Liver.—There were 13 cases of cirrhosis of the liver. Of the 9 patients who were jaundiced, 7 had high transaminase levels and only 2 had normal values. One of the latter was in hepatic coma. Autopsy revealed far-advanced cirrhosis, without evidence of acute necrosis.

Only 1 of the 4 nonjaundiced patients had an elevated serum transaminase level. This patient was found to have a liver abscess and biliary cirrhosis at laparotomy.

Obstructive Jaundice.—There were 10 cases with an obstructive type jaundice; 7 were due to common duct obstruction and 3 were secondary to Thorazine. There was little evidence by laboratory tests of hepatic parenchymal disease in any of these patients. The serum transaminase values in these 10 patients at the height of their icterus were 26, 39, 61, 83, 94, 103, 108, 141, 144, and 268 units. In general, the levels of serum transaminase followed the course of the serum bilirubin.

Comment: Although the majority of these patients with obstructive jaundice had elevated serum transaminases, the levels were lower than observed in the infectious or serum hepatitis group. The observation that normal bile contains approximately 2,000 units per milliliter suggests that bile may be a normal route of excretion of this enzyme and may explain the elevation of serum transaminase in obstructive jaundice without hepatic necrosis.

Carcinomatosis.—There were 10 cases of carcinomatosis. Five of these were shown to have hepatic metastases (four were jaundiced), either by biopsy or post-mortem examination. All 5 had high serum transaminase levels. The remaining 5 cases did not have liver involvement, and all had normal transaminase levels.

Acute Pancreatitis.—The results observed in 16 cases with acute pancreatitis are shown in Table III. Eight of the ten jaundiced patients had elevated transaminase levels, and the other 2 jaundiced patients had values in the borderline range. Four of these jaundiced patients were explored, and the operative findings are noted in Table III. Among the 6 nonjaundiced group, 3 patients had high transaminase levels, 1 border line, and 2 normal.

TABLE III. SERUM TRANSAMINASE ACTIVITY IN 16 CASES OF ACUTE PANCREATITIS

PATIENT	AGE (YR.)	SEX	JAUNDICE	DIASTASE LEVEL*	TRANSAMINASE LEVEL*	REMARKS†
E. B.	54	F	-	700 U.	37 U.	
F. C.	48	M	-	530 U.	52 U.	
M. F.	39	M	-	9000 U.	44 U.	
T. P.	55	M	-	1250 U.	144 U.	
S. R.	66	F	-	1500 U.	53 U.	
M. W.	44	M	-	3600 U.	35 U.	
G. F.	32	M	+	750 U.	44 U.	
A. K.	56	M	+	3000 U.	45 U.	Acute hemorrhagic pancreatitis
A. K.	72	F	+	5140 U.	154 U.	
P. M.	30	F	+	1500 U.	330 U.	Stones in gall bladder, common duct not dilated
T. M.	60	F	+	1125 U.	153 U.	
S. S.	82	M	+	1800 U.	79 U.	
G. S.	64	M	+	1750 U.	329 U.	Empyema of gall bladder, common duct not dilated
H. W.	51	M	+	3000 U.	162 U.	Stones in gall bladder, common duct dilated, no common duct stone
R. Z.	57	F	+	6000 U.	420 U.	
M. T.	55	M	+	1200 U.	180 U.	

*Highest level observed during course of illness.
†Operative findings.

Among the jaundiced patients, serum transaminase levels followed the course of the icterus. There did not appear to be any consistent correlation between the level of serum transaminase and the serum diastase in either the jaundiced or nonjaundiced cases.

Comment: Acute pancreatitis is associated with a high incidence of increased serum transaminase activity. The mechanism or mechanisms for this response remain to be investigated.

Miscellaneous Diseases.—There were 97 cases of miscellaneous diseases observed in this study. Eighty-eight had normal transaminase levels, 2 borderline levels, and 7 high levels.

The 88 patients with normal transaminase levels included the following: acute bacterial pneumonia (9); acute cholecystitis (7); lymphoma (5); chronic uremia (4); essential hypertension (4); cellulitis of leg (3); inactive rheumatic heart disease (3); ureteral calculus (2); psychoneurosis (2); disseminated lupus erythematosus (2); dermatitis (2); severe diabetic acidosis (2); anemia (primary) (2); acute gastroenteritis (2); acute intestinal obstruction (2); pulmonary infarction (2); bronchial asthma (2); pemphigus (2); hydrops of the gall bladder (2); acute cholangitis (1); advanced pulmonary tuberculosis (1); labor (1); brain tumor (1); Charcot-Marie-Tooth syndrome (1); pulmonary fibrosis and emphysema (1); generalized scleroderma (1); perinephritic abscess (1); aortic aneurysm (1); ruptured aortic aneurysm (1); subacute bacterial endocarditis (1); senile brain degeneration (1); trauma to chest (1); salicylate intoxication (1); myotonia congenita (1); acute renal shutdown (1); rheumatoid arthritis (1); osteoarthritis of spine (1); heat exhaustion (1); neurofibroma of abdominal wall (1); postoperative ischial osteotomy (1); cardiac neurosis (1); hemorrhagic cyst of adrenal (1); mesenchymal tumor of omentum (1); duodenal ulcer (1); duodenal ulcer with hemorrhage (1); acute tonsillitis (1); chronic cystitis (1); vascular thrombosis of spinal cord (1).

TABLE IV. PATIENTS OF MISCELLANEOUS GROUP WITH ELEVATED SERUM TRANSAMINASE ACTIVITY

CASE	AGE (YR.)	SEX	SERUM TRANSAMINASE UNITS/ML.	REMARKS
H. I.	43	F	476	Postcommissurotomy syndrome with decompensation and shock. Autopsy revealed anasarca, inactive rheumatic heart disease with fractured stenotic mitral valve, and marked centrilobular liver cell necrosis
H. T.	26	F	670	Granulocytopenia with toxemia and shock. Autopsy revealed maturation arrest of granulocytes, extensive ulcerative tracheobronchitis, and marked liver cell necrosis
L. S.	55	M	106	Following subtotal gastrectomy for pyloric obstruction, developed hypotension and progressive jaundice. Liver biopsy at laparotomy revealed centrilobular liver cell necrosis. Clinically suspected to have acute pancreatitis as well
R. O.	70	F	69	Gangrene of one foot secondary to arteriosclerotic peripheral vascular disease. Developed unexplained shock, persisting for several days
E. B.	21	F	99	Pre-eclampsia in labor
G. F.	67	F	163	Severe right upper quadrant and substernal pain, radiating to both shoulders. One electrocardiogram, normal. Gall bladder, nonfunctioning and containing lithiasis
H. C.	66	M	154	Postoperative for spontaneous rupture of esophagus. Developed empyema, bouts of severe chest pain, and died. No autopsy

The 2 cases with borderline levels included a patient with pernicious anemia in relapse (44 units), and a case of ruptured aortic aneurysm (44 units).

Significant data pertaining to the 7 patients of the miscellaneous group with elevated transaminase levels is summarized in Table IV. In the three instances where pathologic studies were available, the common finding of liver cell necrosis was present.

Comment: The observation of normal serum transaminase levels in 4 patients with chronic uremia and 1 patient with acute renal shutdown indicates, as previously suspected from urinary studies, that deficient renal excretory mechanisms cannot be responsible for the appearance or maintenance of elevated serum transaminase levels.

DISCUSSION

The observations described in this report indicate that serial measurements of serum glutamic-oxalacetic transaminase in patients may be a useful laboratory procedure in a number of disease situations.

Although the glutamic-oxalacetic transaminase is said to be most highly concentrated in heart muscle,[3] clinical observations in patients indicate that serum elevations of this transaminase are not restricted to myocardial necrosis. Experimentally, necrosis of lung, liver, spleen, kidney, and the gastrointestinal tract is also associated with increased serum transaminase activity.[5] The amount of transaminase liberated into the serum probably reflects the amount of tissue involved multiplied by its transaminase concentration. The clinical observations in this report would indicate that the heart and liver commonly are involved sufficiently in disease situations to liberate significant amounts of transaminase. It is quite likely that extensive necrosis of other tissues containing high concentrations of transaminase will also lead to significant rises in the serum level. Studies on patients with obstructive jaundice suggest that elevations in serum transaminase activity may also result from interference with normal excretory pathways. More extensive studies will be required to delineate the usefulness of this serum enzyme assay as a diagnostic laboratory procedure.

SUMMARY AND CONCLUSIONS

1. The serum glutamic-oxalacetic transaminase activity was determined in 400 patients with a variety of diseases.

2. Almost all patients with acute myocardial infarction developed a significantly elevated serum transaminase activity. The peak activity usually appeared twenty-four hours after the onset of pain. Patients with the anginal syndrome did not develop significantly elevated serum transaminase levels.

3. Elevated serum transaminase levels were also observed in patients with hepatic necrosis, as well as the majority of patients with jaundice of any type; acute pancreatitis; and very rapid cardiac arrhythmias.

4. At the present state of knowledge, the serum transaminase assay, when carried out at the appropriate time, is most useful in excluding the presence of significant myocardial or hepatic necrosis.

REFERENCES

1. Cohen, P. P., and Hekhuis, G. L.: Rate of Transamination in Normal Tissues, J. Biol. Chem. **140**: 711, 1941.
2. Karmen, A., Wroblewski, F., and LaDue, J. S.: Transaminase Activity in Human Blood, J. Clin. Invest. **34**: 126, 1955.
3. LaDue, J. S., Wroblewski, F., and Karmen, A.: Serum Glutamic Oxaloacetic Transaminase Activity in Human Acute Transmural Myocardial Infarction, Science **120**: 497, 1954.
4. Wroblewski, F., and LaDue, J. S.: Serum Glutamic Oxalacetic Transaminase as an Index of Liver Destruction and Disease, J. LAB. & CLIN. MED. **44**: 958, 1954 (Abst.).
5. Rudolph, L. A., Dutton, R., and Schaefer, J. A.: Glutamic-oxalacetic Transaminase Levels in Experimental Tissue Damage, J. Clin. Invest. **34**: 960, 1955 (Abst.).

BLOOD ACETYLCHOLINE IN BRONCHIAL ASTHMA

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NUMEROUS investigators have suggested that bronchial asthma is an illness related to dysfunction of the autonomic nervous system manifesting itself by parasympathetic predominance[1-3] Since the pioneering work of Eppinger and Hess,[4] in 1909, studies of the disease states supposedly associated with autonomic imbalance have repeatedly attempted to establish a physiologic basis for such speculations. The investigations of Curry and coworkers,[5-7] a decade ago, supplied further impetus to the search for physiologic proof of such imbalance. They showed that the asthmatic lung is very responsive not only to histamine but to acetyl beta methylcholine (mecholyl) as well. This increased responsiveness was limited to the bronchial tree, as the general systemic reaction to these drugs did not differ from that seen in normal subjects. Stimulated by Curry's work, other workers, including Funkenstein,[8-11] using parenteral epinephrine and intramuscular mecholyl as test drugs in asthmatics in varying feeling states, found that when asthmatic patients were mentally ill and also free of attacks[12] the blood pressure reaction to the test drugs was more of a "sympathetic system response," followed by an induced attack which was mild and was of a relatively short duration. In contrast, when patients were not frankly psychotic and were having asthmatic attacks, the blood pressure reaction was more "parasympathetic" and mecholyl would produce a relatively longer and more severe attack.

In addition to the use of the degree of responsiveness to cholinergic drugs as a means of estimating parasympathetic overactivity, attempts have been made to detect acetylcholine in the circulating blood. Diethelm[13, 14] studied the relationship between various emotions and certain physiologic effects in blood samples drawn at the time of psychiatric interview, and he reported an acetylcholine-like effect correlated with tension and an epinephrine-like effect associated with anxiety, in addition to other effects associated with resentment, guilt, and anger. Vorhaus, Scudamore, and Kark[15] described increased levels of acetylcholine in the venous blood of asthmatic subjects, using the very sensitive clam heart method. However, Ritchie,[16] using a different method, found neither increased cholinergic nor adrenergic properties in venous blood while studying a group of patients with varying psychiatric disorders.

As part of a study of bronchial asthma currently in progress,* an attempt was made to detect acetylcholine or similar substances in venous blood and

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to learn whether the level was significantly different in normal and asthmatic subjects. Previous studies have shown that the level of cholinesterase in bronchial asthma is within normal limits[17]; therefore our studies at this time were restricted to attempts at measuring cholinelike substances.

METHODS

Over a 9-month period, 157 venous blood samples were obtained from 20 men and 8 women patients suffering from various degrees of asthma. Some were symptom-free, some were receiving steroid therapy at the time, and in one instance an asthmatic attack was induced using aerosolized histamine. As controls, 74 samples of blood were obtained from patients without asthma, on the ward or in the outpatient department, and from physicians, employees, and medical students. Samples were drawn between 9 A.M. and 5 P.M. but most frequently at 2 P.M.

Blood was at first collected according to the method of Babkin and associates,[18] but potassium oxalate was found to be satisfactory and this was used in the later studies as an anticoagulant. The samples were drawn from the antecubital veins of all patients, utilizing siliconized equipment in order to minimize trauma to the cellular constituents. The blood was then placed in tubes previously prepared with 1:4,000 Prostigmine and 0.1 N HCL to preserve the acetylcholine present and either heparin or potassium oxalate as an anticoagulant. Whole blood, or plasma or serum, after centrifugation at 2,500 r.p.m. for thirty minutes, was studied within one hour of venipuncture. One milliliter or less of the test sample was added to the clam heart (*Venus mercenaria*) preparation suspended in 10 ml. of sea water, and the tracing was recorded on the smoked drum of a slowly moving mechanical kymograph. The bioassay method has been described by Wait[19] and extensively studied by Welsh and Taub.[20]

The perfusion bath in which the test organ was suspended was constructed so that after each application of either a test sample of blood or a drug the organ could be rapidly washed with fresh sea water, permitting repeated tests with one preparation in the course of a single experiment.

Prior to application of the blood samples, the clam heart was pretreated with certain substances, described below, in an effort to help in the interpretation of the results and to avoid the stimulating effects of 5-hydroxytryptamine, histamine, or epinephrine that might be present.[21] Early in the study, DHE 45 (dehydroergotamine methanesulfonate) was used in a dilution of 0.25 mg. per 100 ml. to stimulate maximally the test preparation, and, later, lysergic acid diethylamide, an even more effective agent, was used in a similar manner.[21] After the test sample had been run in duplicate or triplicate and the tracings recorded, the heart was treated with mytolon chloride (2,5-bis-[3-diethylaminopropylamino]-benzoquinonebis-benzyl chloride), which, in this preparation, has been shown to be the most potent antagonist available for acetylcholine.[22]

In addition, the protein in samples of serum and plasma was coagulated by heat, resuspended in the perfusion fluid, and applied to the test preparation. This was done in order to break up such protein-acetylcholine complexes as might be present. Acetylcholine, known to resist heating, if released, might then be measured. The effect of cholinelike substances on the heart of *Venus mercenaria* is to decrease the amplitude of contraction and to slow the rate.[20] This was the response expected from the application of acetylcholine alone or from blood if it contained acetylcholine or similar substances.

RESULTS

The results and observations in 231 blood samples are described below.

1. Acetylcholine in as small amount as 1×10^{-12} mg. per milliliter, when added to the perfusion fluid by itself or in plasma, serum, or blood (as shown in Fig. 1*A*) gave the expected decrease in rate and amplitude. Boiling acetyl-

choline for five minutes did not affect the response (Fig. 1*B*). The response to acetylcholine added in these various ways was completely blocked by mytolon chloride, as illustrated in Fig. 1*C*.

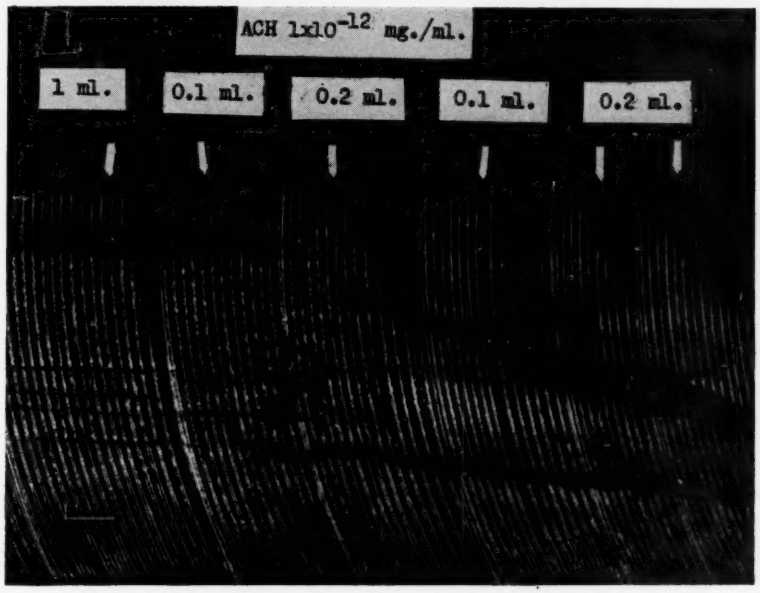

Fig. 1*A*.—The effect of acetylcholine 1×10^{-12} mg. per milliliter in various amounts.

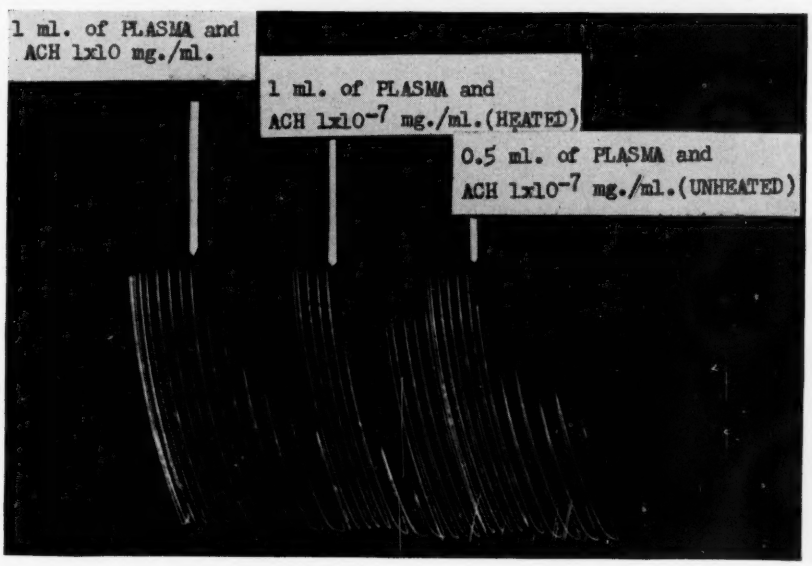

Fig. 1*B*.—The effect of 0.1 ml. acetylcholine 1×10^{-5} mg. per milliliter added to 0.9 ml. of plasma before and after heating.

2. Normal Controls: Among the seventy-four blood samples drawn from the 28 normal controls (19 men and 9 women), there was no instance in which

the typical response (see Fig. 1*B*) was obtained. In certain cases, however, the application of whole blood or separated red blood cells caused a decrease in amplitude of the heartbeat without a significant change in heart rate (Fig. 2*A*). This decrease in amplitude was not blocked by the application of mytolon chloride (Fig. 2*B*) and therefore could not be ascribed to the presence of acetylcholine. On thirty separate occasions, in ten normal subjects, acetylcholine in a concentration of 2 mg. per milliliter was injected at a rate of approximately 20 mg. per minute, until signs and symptoms of flushing, heat, and substernal compression were observed. Bloods were drawn during the period of maximal effect from the antecubital vein of the opposite arm, and in no instance was a *true acetylcholine effect noted* (Fig. 3).

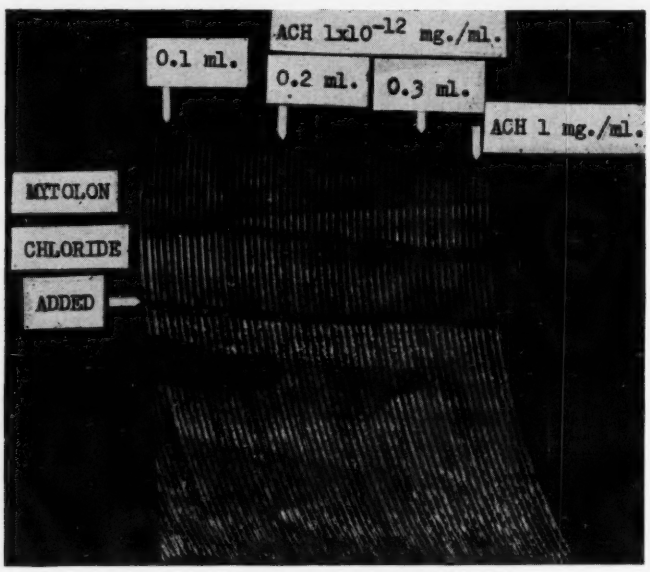

Fig. 1*C*.—The effective inhibition of the acetylcholine response with mytolon chloride.

3. Asthmatic Subjects: One hundred and fifty-seven blood samples in patients with chronic bronchial asthma were obtained. Similar to the demonstration in the control experiments (Fig. 4), no evident cholinelike responses were shown, as demonstrated in Fig. 5. In addition, a single blood sample drawn at the height of a histamine-induced asthmatic attack revealed no acetylcholine effect. Similarly, as was noted in the normal group, an effect on amplitude but not on rate was seen when whole blood or red blood cells were studied. This also was not blocked by mytolon chloride.

In an effort to rule out the cause of any excitation or depression due to substances that were used in the experiments and which might alter the results, a series of tests were made with various substances: Histamine 1×10^{-5} mg. per milliliter, epinephrine 1×10^{-6} mg. per milliliter, potassium chloride 0.004 mg. per milliliter, and a suspension of macerated human platelets were studied. All

caused excitation of the heart preparations. However, this was abolished by pretreatment with lysergic acid diethylamide or DHE 45, and no change in rate or amplitude was then evident. Sodium chloride, 0.9 per cent, 0.05 N sodium

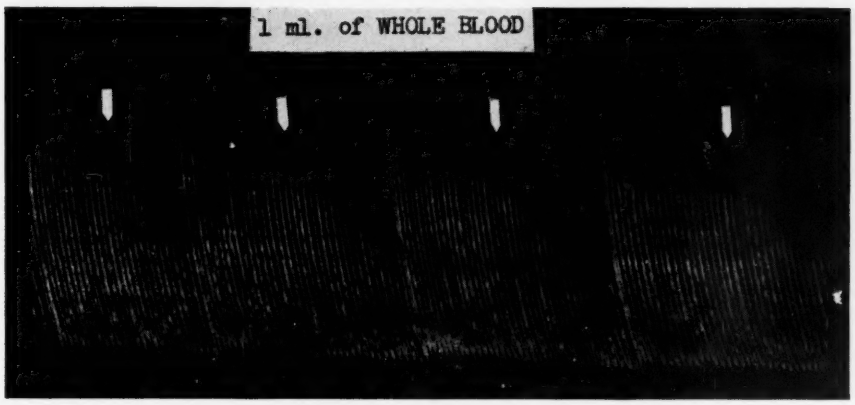

Fig. 2*A*.—The effect of whole blood showing resultant decrease in amplitude, but without comparable decrease in rate.

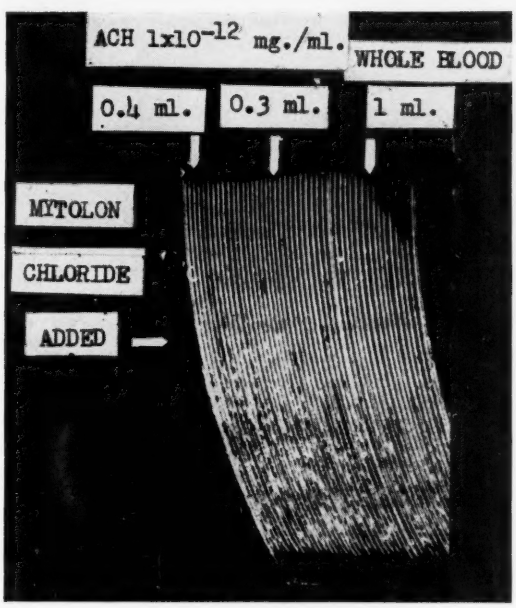

Fig. 2*B*.—The effect of mytolon chloride, showing complete inhibition of acetylcholine, but failure to block the effect of whole blood.

hydroxide, Prostigmine 1:4,000, 0.1 N hydrochloric acid, and dilute potassium oxalate, all used in the procedure, likewise caused no change and thus introduced no difficulty in interpretation.

DISCUSSION

Since 1940 when Prosser[23] first demonstrated the unusual sensitivity of the isolated heart of *Venus mercenaria* to acetylcholine, this preparation has been

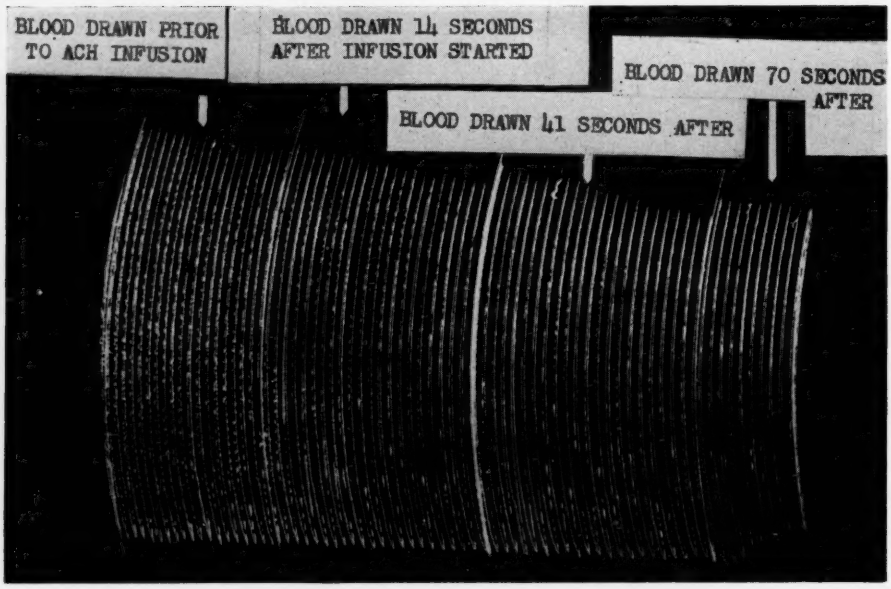
BLOOD DRAWN PRIOR TO ACH INFUSION
BLOOD DRAWN 14 SECONDS AFTER INFUSION STARTED
BLOOD DRAWN 41 SECONDS AFTER
BLOOD DRAWN 70 SECONDS AFTER

Fig. 3.—The effect of plasma drawn prior to and following the slow infusion of 25 mg. of acetylcholine in a concentration of 2 mg. per milliliter.

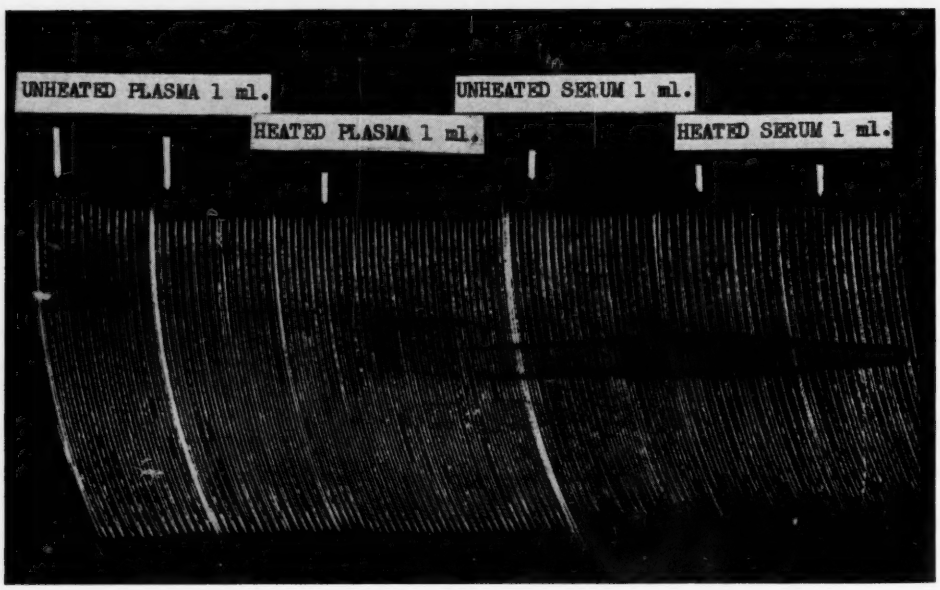

Fig. 4.—The effect of unheated and heated plasma and serum from normal subjects.

extensively employed. In certain respects it is superior to the classical acetylcholine assay preparations such as the dorsal muscle of the leech, the rectus abdominis of the frog, the isolated frog heart, and the blood pressure response of the cat. The advantages of this method, which have been described,[19] are its great sensitivity to acetylcholine; its relative insensitivity to changes in pH, to inorganic ions, and to tissue constituents other than acetylcholine; and the rapidity with which the preparation recovers after washing, which permits repeated determinations in a short space of time. The availability of *Venus mercenaria,* especially in winter months, and the use of natural sea water as the perfusion fluid are additional advantages.

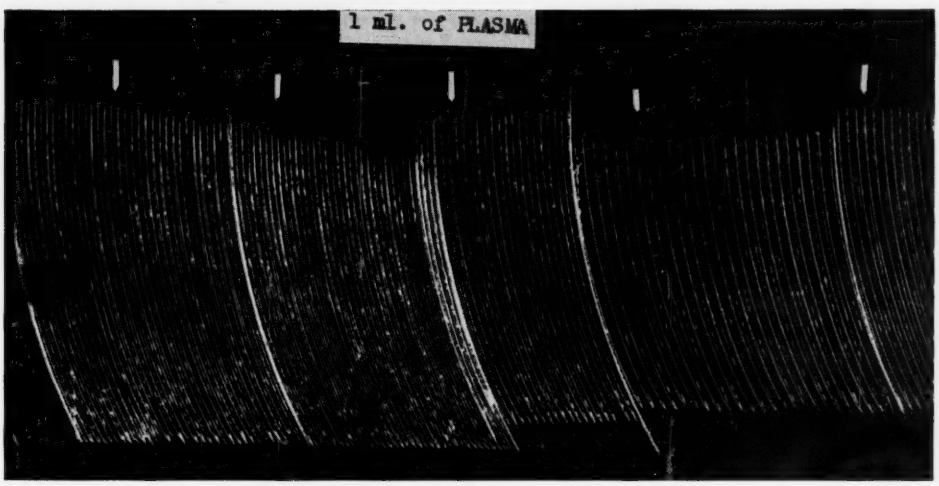

Fig. 5.—The effect of plasma (1 ml.) from 4 patients with bronchial asthma.

It seems evident upon review of the previous work reported on this aspect of bronchial asthma[15] that possibly some of the kymographic evidence presented should not have been ascribed to acetylcholine or cholinelike products. These observations prompted us to use the various chemicals described above in an effort to better delineate the results obtained and to attempt to eliminate all cause for error of interpretation, even though our basic bioassay method was unchanged. It has been shown that epinephrine, norepinephrine, tyramine and histamine are excitatory in relatively high concentration, whereas low concentrations of 5-hydroxytryptamine and ergot alkaloids have a similar effect. Lysergic acid diethylamide contains the indole structure and may be regarded as a complex analogue of 5-hydroxytryptamine. Because it is highly stable and is adsorbed easily to cell surfaces, it stimulates the heart preparation maximally. It thereby eliminates excitatory effects produced by the samples undergoing test, yet it in no way interferes with the depressor response induced by acetylcholine. All the heart preparations, therefore, were treated with either DHE 45 or lysergic acid diethylamide.

Mytolon chloride has proved to be most useful in the work herein described. Substances with two carbonyl groups, such as barbituric acid or

mytolon chloride, apparently inhibit the action of acetylcholine by attaching to the acetylcholine receptor molecule and thereby blocking the nicotinic action of acetylcholine, the only action evident in *Venus mercenaria.*[24] Accordingly, atropine, which blocks only the muscarinic effect, does not inhibit the depressor effect of acetylcholine on the clam heart.[24] Depressor effects which persist after treatment of the clam heart with mytolon chloride, therefore, must be substances other than acetylcholine or cholinelike material. Unless a highly specific blocking agent for acetylcholine such as mytolon is used, difficulties will arise in the interpretation of depressor responses obtained by this method. Thus the depressor effect of red cells or whole blood as observed in our studies, clearly not cholinelike in character, might easily lead to an erroneous conclusion concerning the presence of acetylcholine in the blood. Whether or not this has been a source of confusion in the past is not clear, but it may account for the lack of accord between our results and those of Vorhaus, Scudamore, and Kark.[15] These workers tested serum, not whole blood, but they mention no measures taken to guard against damage to cells which might bring about the release of depressor substances (which the cells appear to contain).

The nature of the acetylcholine-cholinesterase system in the blood is such as to effectively prevent persistence of a cholinelike substance in the blood stream. Were such present, it would undoubtedly manifest itself by symptoms of cholinergic overactivity. Support for this statement will be found in studies in which acetylcholine was given intravenously in doses of 20 mg. per minute. During the period when symptoms of flushing, heat, and chest constriction were evident, plasma obtained from blood drawn from the antecubital vein failed to depress either the rate or amplitude of the clam heart. If circulating acetylcholine could not be demonstrated under these circumstances, it would indeed be unlikely that detectable levels would occur in the absence of overt and extreme parasympathetic overactivity. Stewart has shown that after lethal doses of an effective anticholinesterase (tetraethyl pyrophosphate) in animals, the acetylcholine content of the brain tissue was increased. In one Rhesus monkey, only after overwhelming doses of the agent was acetylcholine measurable in the blood stream.[25] It appears, therefore, that entirely unphysiologic doses are required to bring about measurable acetylcholine levels in the blood by the method used.

SUMMARY AND CONCLUSIONS

1. The blood of 28 asthmatics and 28 normal controls was assayed for acetylcholine on repeated occasions, utilizing as a test organ the isolated heart of the bivalve mollusc *Venus mercenaria.* The advantages of this method are described.

2. Multiple determinations on 231 blood samples failed to show any activity characteristic of acetylcholine in the whole blood, plasma, or serum of asthmatic subjects and normal controls.

3. Whole blood or red blood cells applied to the heart preparation caused a decrease in amplitude of the heartbeat but little change in rate. This effect could not be attributed to acetylcholine.

4. Blood samples drawn from subjects receiving an intravenous injection of a solution of acetylcholine failed to demonstrate any true cholinelike response.

5. Utilizing this bioassay technique, we could not find any humoral evidence of parasympathetic predominance in bronchial asthma.

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REFERENCES

1. Dunbar, F. H.: Emotions and Bodily Changes, New York, 1938, Columbia University Press.
2. French, T. M., and Alexander, F.: Psychogenic Factors in Bronchial Asthma, Psychosomatic Medicine Monographs Vols. 1 and 2, 1941.
3. Fromm-Reichmann, F.: Zur psychopathologie des asthma bronchiale, Med. klin. **18**: 1066, 1922.
4. Eppinger, H., and Hess, L.: Zur Pathologie des Vegatativen Nervensystems, Ztschr. f. klin. Med. **67**: 345, 1909.
5. Curry, J. J.: Comparative Action of Acetyl-Beta-Methyl Choline and Histamine on the Respiratory Tract in Normals, Patients With Hay Fever, and Subjects With Bronchial Asthma, J. Clin. Invest. **26**: 430, 1947.
6. Curry, J. J., Fuchs, J. E., and Leard, S. E.: Clinical Implication of Effect of Various Anticholinergic Agents in Modifying Pulmonary Response of Asthmatic Subjects to Injected Methacholine, Bull. New England M. Center **10**: 164, 1948.
7. Curry, J. J., and Leard, S. E.: The Action of Pilocarpine on Lungs in Normal and Asthmatic Subjects, J. Lab. & Clin. Med. **33**: 585, 1948.
8. Funkenstein, D. H., Greenblatt, M., and Solomon, H. C.: Autonomic Nervous System Changes Following Electric Shock Treatment, J. Nerv. & Ment. Dis. **108**: 409, 1948.
9. Funkenstein, D. H., Greenblatt, M., and Solomon, H. C.: Psychophysiological Study of Mentally Ill Patients, Am. J. Psychiat. **106**: 16, 1949.
10. Funkenstein, D. H., Greenblatt, M., and Solomon, H. C.: Psychophysiological Study of Mentally Ill Patients, part II, Am. J. Psychiat. **106**: 116, 1949.
11. Funkenstein, D. H., Greenblatt, M., and Solomon, H. C.: Autonomic Changes Paralleling Psychologic Changes in Mentally Ill Patients, J. Nerv. & Ment. Dis. **114**: 1, 1951.
12. Funkenstein, D. H.: Variation in Response to Standard Units of Chemical Agents During Alterations in Feeling States in Relation to Occurrence of Asthma, A. Res. Nerv. & Ment. Dis., Proc. **29**: 566, 1950.
13. Diethelm, O., Fleetwood, M. F., and Milhorat, A. T.: The Predictable Association of Certain Emotions and Biochemical Changes in the Blood, A. Res. Nerv. & Ment. Dis., Proc. **29**: 262, 1950.
14. Diethelm, O., Doty, E. J., and Milhorat, A. T.: Emotions and Adrenergic and Cholinergic Changes in the Blood, Arch. Neurol. & Psychiat. **54**: 110, 1945.
15. Scudamore, H. H., Vorhaus, H. J., and Kark, R. M.: Acetylcholine and Cholinesterase in the Blood of Patients Suffering With Bronchial Asthma, J. Lab. & Clin. Med. **37**: 860, 1951.
16. Ritchie, J. A.: Adrenergic and Cholinergic Properties of Blood, J. Nerv. & Ment. Dis. **114**: 307, 1951.
17. Hawes, R. C., and Alles, G. A.: Cholinesterase in the Blood of Allergic Patients, J. Allergy **12**: 1, 1940.
18. Babkin, B. P., Schacter, M., Dworkin, S., and Rosenberg, G. M.: The Preservation in Vitro of Acetylcholine in Whole Blood, Rev. canad. biol. **1**: 761, 1942.
19. Wait, R. B.: The Action of Acetylcholine on the Isolated Heart of Venus mercenaria, Biol. Bull. **85**: 79, 1943.
20. Welsh, J. H., and Taub, R.: The Action of Choline and Related Compounds on the Heart of Venus mercenaria, Biol. Bull. **95**: 346, 1948.
21. Welsh, J. H.: Excitation of the Heart of Venus mercenaria, Arch. exper. Path. u. Pharmakol. **219**: 23, 1953.
22. Luduena, F. P., and Brown, T. G., Jr.: Mytolon and Related Compounds as Antagonists of Acetylcholine on the Heart of Venus Mercenaria, J. Pharmacol. & Exper. Therap. **105**: 232, 1952.
23. Prosser, C. L.: Acetylcholine and Nervous Inhibition in the Heart of Venus mercenaria, Biol. Bull. **78**: 92, 1940.
24. Welsh, J. H., and Taub, R.: Structure-Activity Relationships of Acetylcholine and Quarternary Ammonium Ion, J. Pharmacol. & Exper. Therap. **99**: 334, 1950.
25. Stewart, W. C.: Accumulation of Acetylcholine in Brain and Blood of Animals Poisoned With Cholinesterase Inhibitors Brit. J. Pharmacol. **7**: 270, 1952.

COMPARATIVE STUDIES WITH 18 STRAINS OF HISTOPLASMA

Morphology in tissues and virulence of African and American Strains

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IN VIEW of the lack of pertinent data, it was felt that a large number of strains of Histoplasma should be compared. In general, mice are used for virulence studies, but it was shown that the hamster is much more susceptible than the mouse.[8, 8c, 13, 14] Quantitative data were for this reason obtained in comparing the virulence in hamsters and mice. The use of an animal which would constantly and rapidly develop lethal disease is very important for evaluation of therapeutic results. The hamster seems to offer such conditions.[8c] Little was known about the comparative pathology of experimental histoplasmosis since many different strains and two different animal species were used.

Another point which induced us to start this investigation was the desire to clarify whether an African species could be separated from the classical *Histoplasma capsulatum* by morphologic and biologic methods, since histoplasmosis with large forms was reported in African human and animal cases.[3, 3a, 4, 5, 10, 17, 19]

Eighteen different strains of Histoplasma were available for cultural and virulence studies. The source of the strains was as given in Table I.

METHOD

All strains were maintained on Sabouraud's glucose agar and transformed on Kurung's egg medium into the yeast phase. After ten days, suspensions were made in 0.85 per cent physiologic saline solution and hamsters and mice were injected within a few hours. Counts were made in the hemocytometer, and a final dilution of 40 to 60 million of yeast cells per milliliter was obtained. All animals were injected intraperitoneally with 0.5 ml. of the suspended yeast cells. The initial weight of the male hamsters was 25 to 30 grams, of the male Swiss white mice, 18 to 20 grams. The animals were kept in glass jars in groups of 5. After eight weeks the suriving animals were sacrificed, cultures and vital organs for microscopic study were taken. The organs were fixed in 10 per cent Formalin and stained with hemotoxylin-eosin and Gridley stain,[12] routinely. The animals found dead during the experiment were treated the same way.

RESULTS

The mortality rate of hamsters and mice showed a striking difference. Graph I gives information on comparative mortality between mice and ham-

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sters. Graph II shows results obtained with strains 27 and 19 on hamsters. Strain 407 (*Histoplasma farcinimosum*), the 18th strain, was not lethal to any of the animals.

TABLE I. MORPHOLOGIC CHARACTERS OF 18 STRAINS OF HISTOPLASMA USED IN THIS STUDY

	ORIGIN AND SOURCE OF STRAIN	MYCELIAL PHASE* SPORES		YEAST PHASE† CELLS	
		TUBERCULATE	MICRO	SMALL	LARGE
	American				
5	"Soil" water tower, Cincinnati	+++	++	+++	±
18	Acute lethal human histoplasmosis, Cincinnati	+	+++	+++	+
19	Subacute lethal human histoplasmosis, Cincinnati	+	+++	+++	+
20	Dog, arrested pulmonary foci, Cincinnati	+	+++	+++	±
27	Dog, disseminated lethal disease, Cincinnati	++	+++	+++	±
31	Chronic cavitary pulmonary histoplasmosis, Cincinnati	++	++	+++	+
35	Chronic cavitary pulmonary histoplasmosis, Cincinnati	+++	+++	+++	±
63	Chronic cavitary pulmonary histoplasmosis, Cincinnati	++	++	+++	±
65	Subacute lethal human histoplasmosis, Cincinnati	+++	+++	+++	±
69	Pulmonary (benign) infiltrate, Cincinnati	+++	+	+++	0
6617	Spotted skunk, Georgia (Dr. Emmons)	±	++	++	++
6621	Human strain, Georgia (Dr. Emmons)	±	+++	++	++
2764	Soil, Virginia (Dr. Emmons)	+++	±	+++	0
	African				
261	Generalized human chronic histoplasmosis, Africa	+	++	+++	+
6522	London School Hyg. & Trop. Med., human case, Africa (Chertsey)	+	++	+++	+
6625	London School Hyg. & Trop. Med., human case, Africa (Ross)	+	++	+++	++
44	Human localized histoplasmosis from Africa (Dr. Vanbreuseghem)	+	++	++	++
407	*H. farcinimosum* from Burma via London, School Hyg. & Trop. Med.	0	+++	++	++

*Grown on Sabouraud's dextrose agar and corn meal agar.
†Grown on Kurung's egg medium and blood cystin agar.
± very few cells
+ few cells
++ numerous cells
+++ very numerous cells

Without exception, hamsters proved to be much more susceptible to the infection with Histoplasma. Great variations were found between the different strains, apparently not related to the source of the organisms. Two soil strains (5,2764) were lethal far faster to hamsters than some strains isolated from disseminated human cases (18, 19). On the other hand, several strains of *Histoplasma capsulatum* (35,44,261,6625) were not lethal at all. However, even without producing death, disease was present in all hamsters, as proved by culture and microscopic examination of tissues. This was not the case in all mice.

Table II gives information on the presence of disease in the organs of the inoculated animals on microscopic examination of liver, spleen, lung, and heart. It also informs on the presence of organisms in tissues on microscopy and on the results of cultures taken from liver and spleen. The difference in mortality

already delineated in Graph I becomes more impressive when it is seen that the inflammatory changes in the hamsters are very much more pronounced than in mice. The same holds true with positive cultures, even if cultures are frequently positive in animals which show little mortality and disease. Some of the African strains failed to produce disease in mice and the same was true with the American strains 31 and 6621. *H. farcinimosum* (strain 407) produced minimal reaction of tuberculoid character in the peritoneum of one mouse and two hamsters. Organisms were numerous in these "tubercles," but only one positive culture was obtained from a hamster.

A considerable surprise was the frequent finding of large forms of Histoplasma in the tissues, which formerly was thought to occur only in African strains, and which had induced Vanbreuseghem to suggest the creation of a new species, *Histoplasma duboisii*.[19]

TABLE II. RESULTS OF INOCULATION OF MICE AND HAMSTERS WITH HISTOPLASMA

	MICE				HAMSTERS				
STRAIN	MORTALITY*	DISEASE	ORGANISMS	CULTURE†	MORTALITY	DISEASE	ORGANISMS	CULTURE	LARGE FORMS
5	3/5	+++	+++	5/5	5/5	+++	+++	5/5	±
18	0/5	(±)	(±)	5/5	2/5	+++	+++	5/5	±
19	0/5	(±)	(±)	5/5	5/5	++	+++	5/5	++
20	0/5	+++	+++	5/5	5/5	+++	++	5/5	±
27	1/5	+++	++	5/5	25/25	+++	+++	25/25	±
31	0/5	–	–	5/5	5/5	+++	+++	5/5	++
35	0/5	(±)	(±)	3/5	0/5	++	+	5/5	++
63	0/5	(±)	(±)	5/5	4/5	++	++	5/5	±
65	2/5	++	++	5/5	5/5	+++	+++	5/5	*
69	0/5	+	±	3/5	5/5	+++	+++	5/5	++
6617	0/5	++	+	5/5	5/5	+++	+++	5/5	++
6621	3/5	–	–	5/5	5/5	+++	+++	5/5	++
2764	1/5	+++	+++°	5/5	5/5	++	++	5/5	±
261	0/5	–	(±)	0/5	0/5	++	++	5/5	++
6522	0/5	–	–	3/5	4/5	++	+	5/5	+
6625	0/5	–	–	4/5	0/5	±	±	4/5	–
44	0/5	–	(±)	0/5	0/5	±	(±)	1/5	+
407	0/5	(±)	(±)	0/5	0/5	(±)	(±)	1/5	–

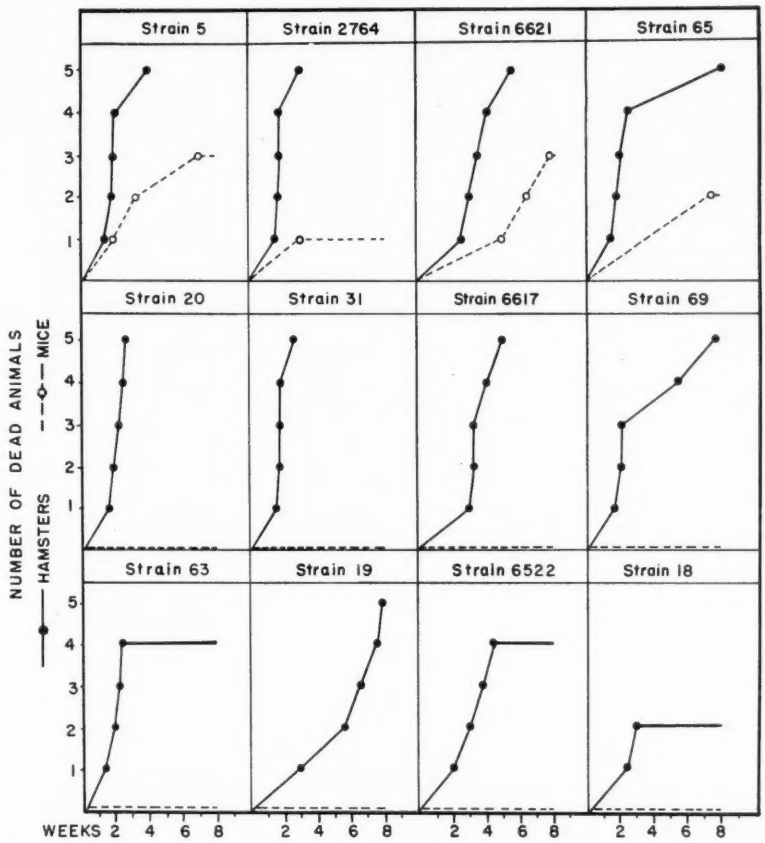
Liver, spleen, lung, and heart were examined. The sections were stained with hematoxylin-eosin and with the Gridley stain. Generally liver and spleen gave a fair idea about the severity of the infection.

o—smooth cells with thin wall 6-8 μ in size.
(±)—minimal isolated lesions or extremely rare organisms.
±—minimal lesions or rare organisms.
+—moderate number of lesions or organisms.
++—extensive lesions or goodly number of organisms.
+++—severe diffuse lesions or very numerous organisms.

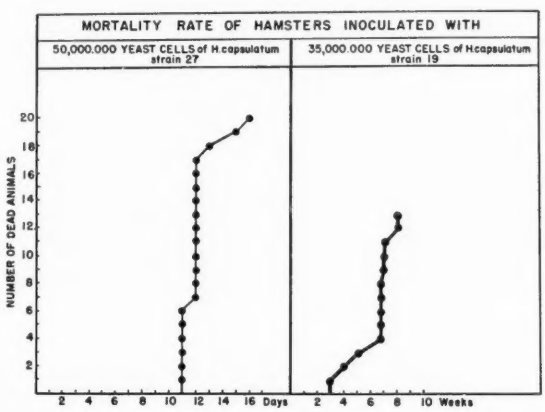
$$\frac{\text{Nominator}}{\text{Denominator}} = \frac{\text{*Number of dead animals}}{\text{Total number of animals}} \text{ or } \frac{\text{†Positive cultures}}{\text{Total number of cultures}}$$

The large forms were found in the majority of strains in hamsters but in only one mouse. They occurred in great variation in reference to size, shape, and staining properties.

These forms measure from 6 to 27μ and stain in a very variable way with different dyes. With hematoxylin, sometimes uniformly deep-blue bodies without structure are seen. In other forms, a laminated onionlike picture is ob-



Graph I.—Comparative virulence studies with 16 strains of histoplasma in hamsters and mice. The strains 35, 44, 261, and 6625 did not produce lethal infection in either animal species.


MORTALITY RATE OF HAMSTERS INOCULATED WITH
50,000,000 YEAST CELLS of H.capsulatum strain 27
35,000,000 YEAST CELLS of H.capsulatum strain 19
NUMBER OF DEAD ANIMALS
2 4 6 8 10 12 14 16 Days
2 4 6 8 10 Weeks

Graph II.—Strain 27 produced mortality in 100 per cent of the inoculated animals. Strain 19: 13 of 15 hamsters inoculated died within 8 weeks.

Fig. 1.

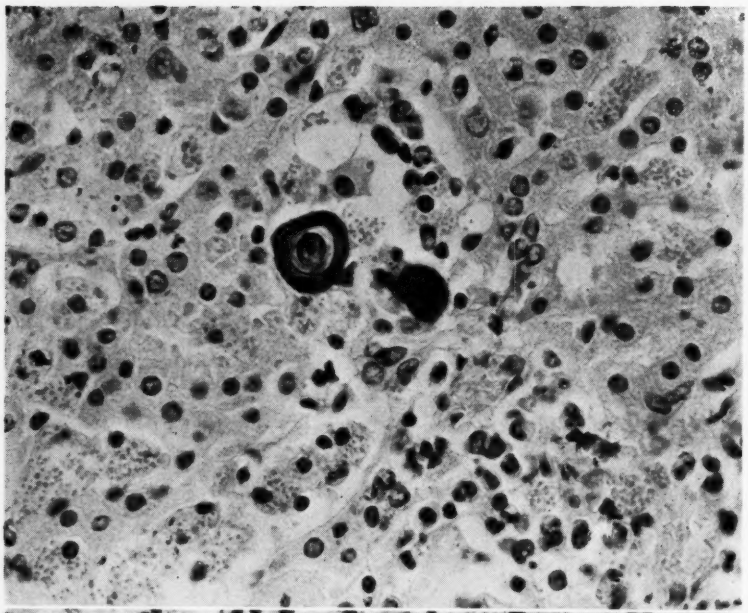


Fig. 2.

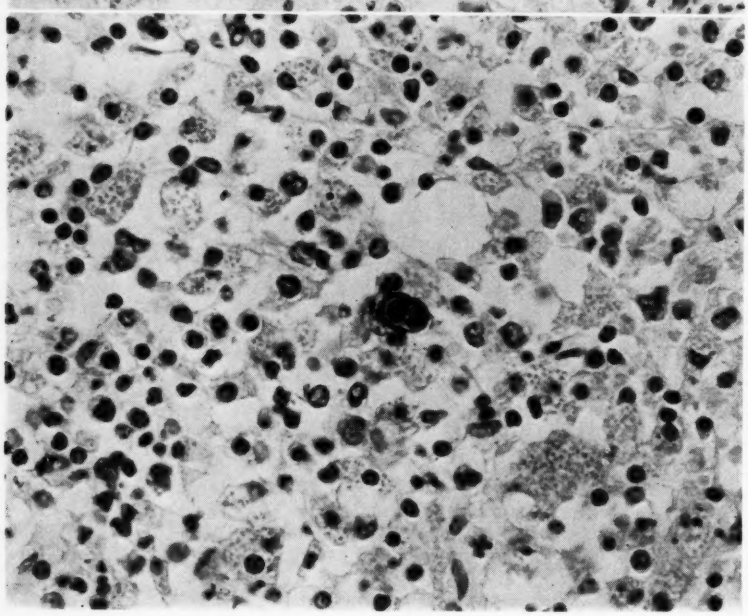


Fig. 1.—Strain 19. Liver of hamster. Numerous small forms in histiocytes and two large forms. One appears as a dark undifferentiated dot; the other shows the laminated structure characteristically found in this animal species after inoculation with American and African strains. (Hematoxylin-eosin, ×650; reduced ¼. Isolate from Cincinnati, Ohio.)

Fig. 2.—Strain 19. Spleen same animal as before. Numerous small forms in swollen histiocytes. In the center, a large form, apparently budding, with a capsule which follows the outline of the indented organism. Notice similarity to *B. dermatitidis*. (Hematoxylin-eosin, ×650; reduced ¼.)

Fig. 3.

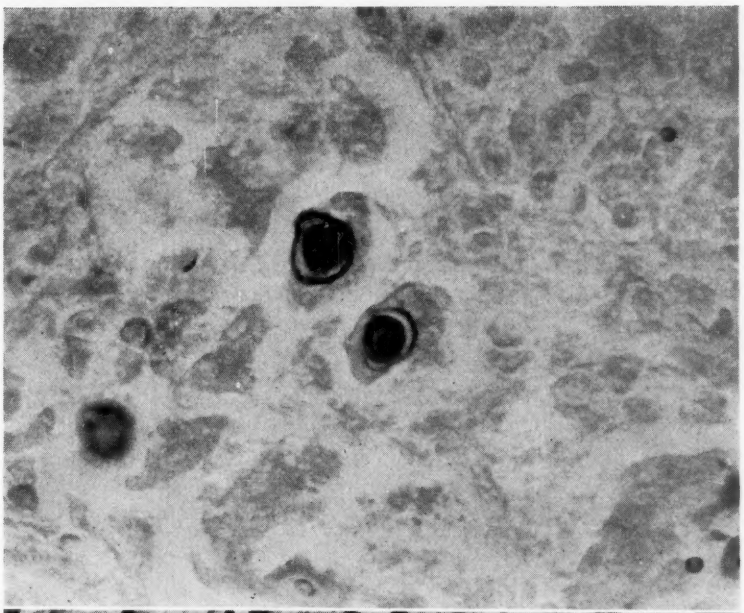


Fig. 4.

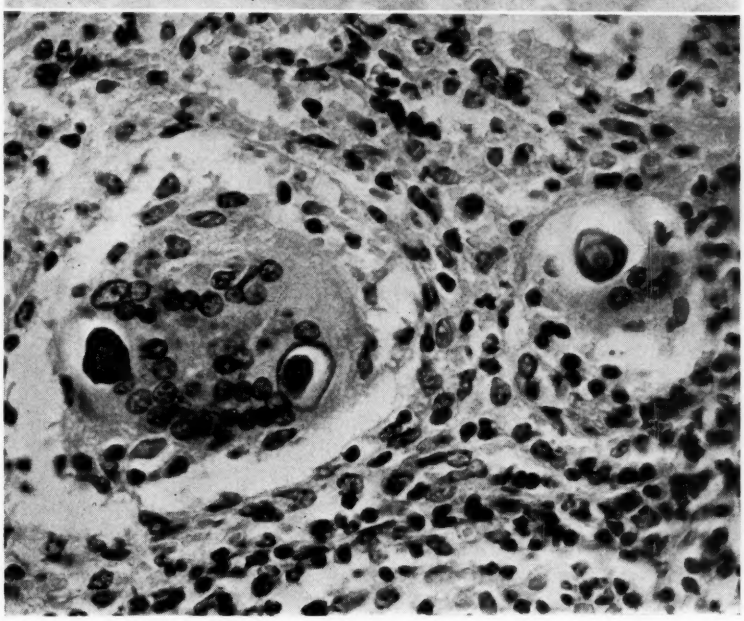


Fig. 3.—Strain 18. Liver, in every respect identical large forms, produced in hamsters by American strains. Gridley stain. Notice the small forms in the periphery close to the right border, isolate from Cincinnati, Ohio. (×950; reduced ¼.)

Fig. 4.—Strain 44. Large forms in giant cell in tubercular structure in the spleen of hamster. Notice the concentric arrangement of the organisms. Hematoxylin-eosin, isolate from Africa. (×650; reduced ¼.)

Fig. 5.

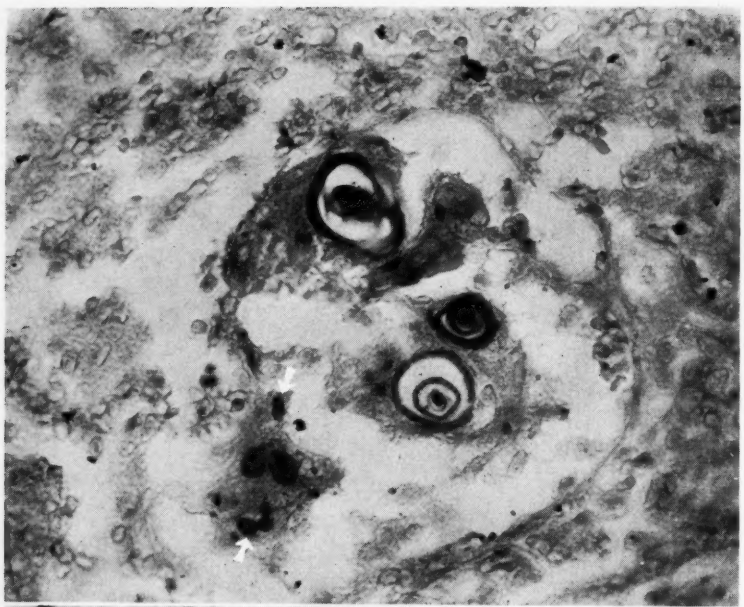


Fig. 6.

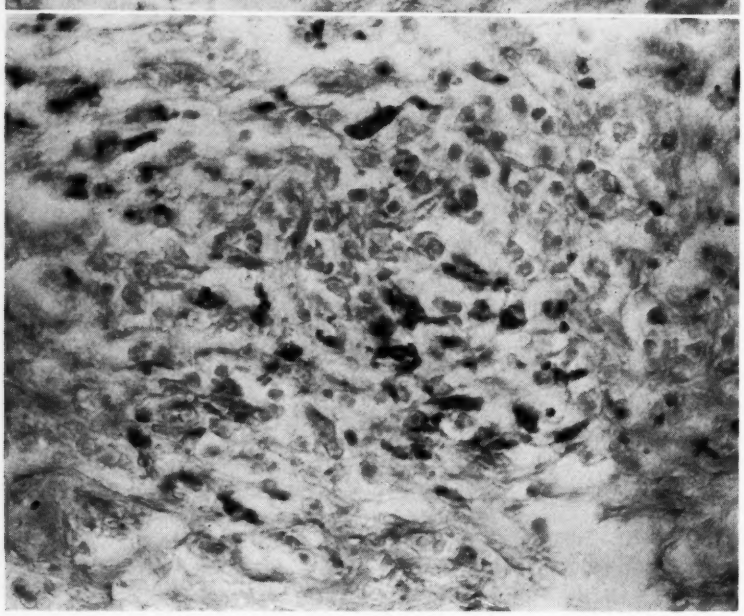


Fig. 5.—Strain 44. Tuberculoid nodule in spleen. Three very large forms of the organisms are in the center and show typical onionlike structure. In the center, small cells are seen which in size and shape and in view of buds seem to represent yeast cells. The concentric structures may possibly represent a capsule. Small forms are scattered through the nodule (arrows). Two large forms with gelatinous-appearing envelope, left lower half of picture. (×650; reduced ¼.)

Fig. 6.—Strain 44. The same strain which produces large forms in the hamster has exclusively small forms in the mouse. Gridley stain. (×650; reduced ¼.)

Fig. 7.

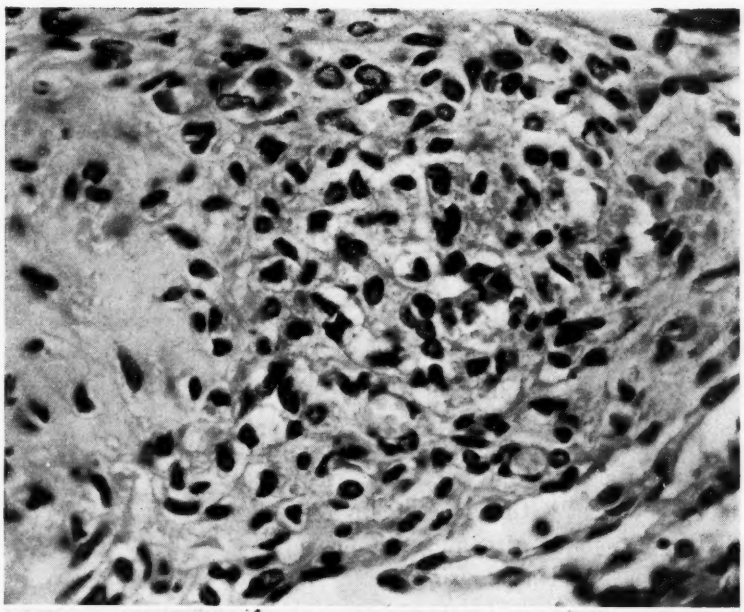


Fig. 8.

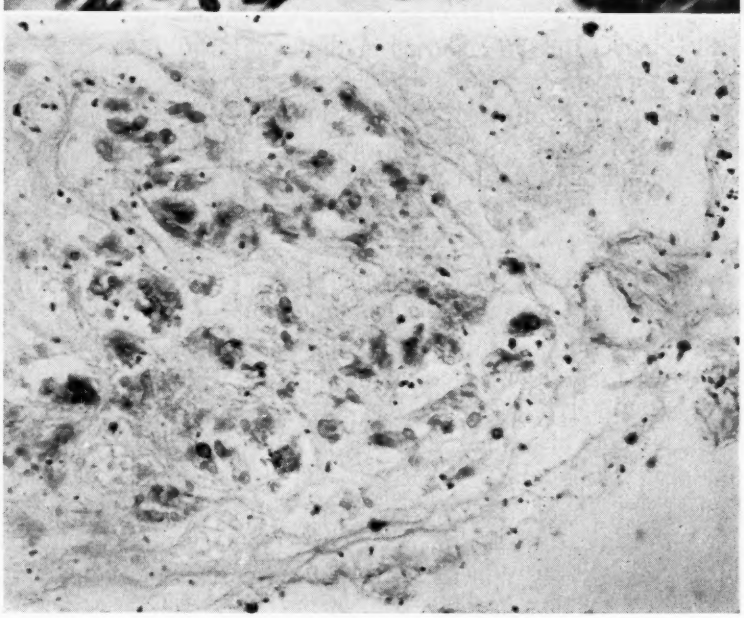


Fig. 7.—Strain 407. *H. farcinimosum* produced small histiocytic nodules in the peritoneum of a mouse. Hematoxylin-eosin. (×650; reduced ¼.)

Fig. 8.—Strain 407. (*H. farcinimosum*) small yeast cells are found with the Gridley stain in tuberculoid nodule in the splenic hilus shown in Fig. 7.

served. Buds sometimes seem to be present. In the laminated structures, small bodies are often seen in the center; they have the approximate size of 2 to 5μ and are either round or ovoid in shape and sometimes seem to be budding. With the Gridley stain,[12] the separate layers are much more differentiated and generally stained pale purple. Sometimes the entire structure has an opaque, slightly pinkish appearance and in such forms no lamination is seen.

These large forms were found especially in the liver and in intraperitoneal exudate. In the spleen and other organs, they were only rarely seen. Some of the large cells are solitary; others occur in groups; some are found in giant cells or within a granulomatous lesion; others are lying in the tissue, apparently producing little reaction. Frequently, small forms of typical size and shape are found in the same section or microscopic field. The number of large forms varies from rare ones, discovered only after extensive search, to huge amounts.

Comparison of the large forms seen in a human case from Africa with the cells seen in hamsters on the one hand, and comparison of the forms obtained in hamsters inoculated with African and American strains show identical features as far as size, shape, and staining properties go. Attempts failed to separate morphologically the strains from the appearance in tissues.

Parallel experiments with mice which were injected at the same time and with the same yeast cell suspension as the hamsters were entirely negative as far as production of large forms go, except for one mouse inoculated with strain 5 which had a few large forms in the liver. All strains produced small "classical" Histoplasma yeast cells in mice and hamsters, including the African strains (6522,6625,261, and 44). Large forms were found in the livers of hamsters inoculated with 13 different strains, in 8 spleens and in the peritoneum of 10. In the lungs, large cells were exceptional (Figs. 1-8).

DISCUSSION

Several observations seem of considerable interest. The hamster proved to be an animal which was much more susceptible to histoplasmosis than the mouse. Mortality occurs within a relatively short period, which renders this animal ideal for use in therapeutic experiments. It was not unusual to find 5 or more dead animals in one day (compare Graph II).

The hamster was also almost the sole producer of large forms which had been heretofore associated only with African strains. Catanei,[3, 3a] in 1945, was the first to describe large cells of 6 to 12.5μ in a subcutaneous abscess in an African Negro. Subsequently, several other reports[4, 9, 10, 17] appeared and in 1952 Vanbreuseghem[19] introduced a new species which he called *H. duboisii*. All these African strains are identical with *Histoplasma capsulatum* in the mycelial phase, grossly and microscopically. The yeast phase, however, contains large cells in addition to the "classical" small forms.

However, this in our experience[8b] is not an exclusive characteristic of the African strain. We have observed variable amounts of large cells in the yeast phase of American strains isolated from patients from different parts of the United States.

In tissues, we found large forms in hamsters inoculated with American and African strains. Frequently, the number of large forms in animals inoculated with American strains was enormous. Large forms of *Histoplasma capsulatum* have been occasionally observed in the tissues of American patients[1, 2, 7, 15, 18]

In tissue explants, large forms were obtained (up to 20μ) which had, in general, thinner walls than the forms seen in the hamsters of our experiment.[16] However, isolated large forms were seen in the explants which would be indistinguishable from the organisms seen in hamsters or in tissues from African patients. On the other hand, large forms with thin walls were also occasionally observed in tissues of hamsters and mice.

The presence of large forms in tissues of non-African patients and the experimental production of such forms indicate the great variability of the parasitic phase of *Histoplasma capsulatum*.

The mycelial phase is quite stable and shows only differences in the quantitative relationship between macro and microspores. In the yeast phase, however, large variations of size (1μ to 17μ) and shape (round to ovoid to pyriform) are found. This in part may indicate the tendency of the parasitic phase to convert into the more stable mycelial phase.

Histoplasma capsulatum shows considerable variation of morphologic and biologic characteristics. In the mycelial phase, great differences in the number of specific spores were observed.[6] In the yeast phase, likewise, the proportion of large to small forms varies but does not seem to indicate the need for separation of a distinct species.[8b]

Mortality of hamsters was obtained with some African strains (in this experiment, strain 6522, formerly with strain 261)[8a] whereas other strains were not lethal but produced disease (strains 44,6625) in hamsters. Here it must be mentioned that one American strain (35) did not produce lethal disease.

Histoplasma farcinimosum (S407) did not produce tuberculate spores, grew very slowly, and required the presence of amino acids. In the yeast phase, it was not lethal to either hamsters or mice, but small granulomatous lesions containing small yeast cells of about 2μ size were found in the peritoneum of one mouse and two hamsters.

Obviously isolated large forms (without accompanying small "classical" cells) may lead to differential diagnostic considerations. *Blastomyces dermatitidis* and, to lesser degree, *Cryptococcus neoformans* can be mistaken for such forms in tissues. On the other hand, "dual" infections may represent just variation in size of Histoplasma.[20] The importance of cultural proof can be hardly overemphasized.

SUMMARY

1. A comparison of American and African strains of *Histoplasma capsulatum* revealed great variations not only in the morphology and growth rate but also in virulence.

2. Mortality was always much more pronounced in hamsters; of 17 strains of *Histoplasma capsulatum,* 13 killed hamsters after intraperitoneal injection of the yeast phase with generalized infection. Only 4 strains produced death in mice under the same conditions.

3. *Histoplasma farcinimosum* was not lethal to any of the animals but produced small "tubercles" in the peritoneum of animals.

4. Death in hamsters inoculated with virulent strains occurs in a relatively short time as a consequence of disseminated disease, which makes this species very desirable for therapeutic experiments.

5. Of 13 American strains of *H. capsulatum,* only one was not lethal for hamsters in the conditions of these experiments; of 4 African strains, only one was lethal but all produced disease in hamsters.

6. Highly virulent strains (5,20,27,31,63,2764) killed animals within three weeks, but other strains required considerably more time or were not lethal at all.

7. Large forms measuring 6 to 27μ, similar to the organisms observed in African human and animal histoplasmosis were found in 12 American and 3 African strains in hamsters.

8. From this comparative study, it is concluded that *Histoplasma capsulatum* occurs with great variation of morphology, cultural characteristics, and virulence; at present there seems to be no safe way to recognize a separate species.

REFERENCES

1. Almeida, F. de: Consideracao sobre as formas grandes do H. capsulatum nos tecidos, Folia Clin. Biol. Sao Paolo **20:** 183-190, 1953.
2. Binford, C. H.: Histoplasmosis. Tissue Reactions and Morphologic Variations of the Fungus, Am. J. Clin. Path. **25:** 25-36, 1955.
3. Catanei, A.: Résultats de l'étude du pouvoir pathogène d'une souche soudanaise d'Histoplasma capsulatum, Arch. Inst. Pasteur d'Algérie **23:** 260-268, 1945.
3a. Catanei, A., and Kervran, P.: Nouvelle mycose humaine observée au Soudan francais, Arch. Inst. Pasteur d'Algérie **23:** 169-172, 1945.
4. Clarke, G. H. V., Walker, J., and Winston, R. M.: African Histoplasmosis. J. Trop. Med. **56:** 277-280, 1953.
5. Courtois, G., Segretain, G., Mariat, F., and Levaditi, J. C.: Mycose cutanée à corps levuriformes observée chez les singes africains en captivité, Ann. Inst. Pasteur, 1955 (in press).
6. Cozad, G. C., and Furcolow, M. L.: Laboratory studies of *H. capsulatum.* II. Size of the spores, J. Inf. Dis. **92:** 77-84, 1953.
7. Crumrine, R. M., and Kessel, J. F.: Histoplasmosis (Darling) without splenomegaly, Am. J. Trop. Med. **11:** 435-449, 1931.
8. Drouhet, E., and Segretain, G.: Histoplasmose expérimentale chez le hamster doré, Ann. Inst. Pasteur **88:** 381-383, 1952.
8a. Drouhet, E.: Unpublished data.
8b. Drouhet, E., and Schwarz, J.: Growth and Morphology of Histoplasma capsulatum in the Mycelial and Yeast Phase. Study of 18 Strains of American and African Origin. Ann. Inst. Pasteur. (in press).
8c. Drouhet, E., and Schwarz, J.: The Action of Nystatin (Mycostatin) on *Histoplasma capsulatum* In Vitro and in Hamsters and Mice. (in press) Antibiotics and Chemother.
9. Dubois, A., Janssens, P. G., Brutsaert, P., and Vanbreuseghem, R.: Un cas d'histoplasmose africaine, Ann. Soc. belge de med. trop. **32:** 559-583, 1952.
10. Duncan, J. T.: A Unique Form of Histoplasma, Tr. Roy. Soc. Trop. Med. & Hyg. **40:** 364-365, 1946.
11. Emmons, C. W., Morlan, H. B., and Hill, E. L.: Histoplasmosis in Rats and Skunks in Georgia, Pub. Health Rep. **64:** 1423-1430, 1949.

12. Gridley, M. F.: A Stain for Fungi in Tissue Sections, Am. J. Clin. Path. **23**: 303-307, 1953.
13. Hazen, E. L., and Tahler, E. D.: Experimental Histoplasmosis of Skin and Mucous Membranes in Rabbits, J. Invest. Dermat. **15**: 205-213, 1950.
14. McLeod, J. H., Emmons, C. W., Ross, S., and Burke, F. G.: Histoplasmosis; a Report of 4 Cases, Two in Siblings. Histoplasmin Test and Other Diagnostic Procedures, J. Pediat. **28**: 275-295, 1946.
15. Parson, R. J., and Zarafonetis, C. J. D.: Histoplasmosis in Man, Arch. Int. Med. **75**: 1-23, 1945.
16. Schwarz, J.: Giant Forms of Histoplasma capsulatum in Tissue Explants, Am. J. Clin. Path. **23**: 898-903, 1953.
17. Silvera, W. D., and Konstam, P. G.: A Case of Histoplasma Duboisii Infection, West African M. J. **3**: 32-37, 1954.
18. Silverman, F. N., Schwarz, J., Lahey, M. E., and Carson, R.: Histoplasmosis, Am. J. Med. **19**: 410-459, 1955.
19. Vanbreuseghem, R.: Histoplasma Duboisii and African Histoplasmosis, Mycologia **45**: 803-816, 1953.
20. Weed, L. A.: Large and Small Forms of Blastomyces and Histoplasma. Editorial, Am. J. Clin. Path. **23**: 921-923, 1954.

LABORATORY METHODS

THE BIOLOGIC ASSAY OF L-METHIONINE USING A MUTANT OF BACT. COLI STRAIN K-12

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THE liver plays an important part in protein and amino acid metabolism. The function of methionine in experimental hepatic necrosis (Himsworth[1]) and its metabolism in patients with liver disease (Kinsell and associates[2]) have been investigated particularly carefully. Many patients with liver disease have a high fasting plasma methionine level and are unable to metabolize methionine. In this paper, a new method for the estimation of L-methionine in plasma is described employing a methionine-requiring mutant of *Bacterium coli* Strain K-12.

Certain mutant strains of *Bact. coli* K-12 (mutants 58/161 and J^{5-3}) employed in work on genetic recombination, unlike typical *Bact. coli* strains, cannot grow in a basic synthetic medium containing only glucose and ammonium salts as source of carbon and nitrogen, respectively. Mutant 58/161 requires L-methionine and mutant J^{5-3} requires both L-methionine and L-proline for growth, and these amino acids must be added to the medium. Since as little as 5 μg per milliliter methionine promotes good growth of the mutants in synthetic fluid medium, whereas 0.5 μg per milliliter still allows the development of a visible turbidity, these mutants appeared to be suitable for the estimation of methionine levels in the blood. With limiting concentrations of methionine, the amount of growth (judged nephelometrically) is proportional to the methionine concentration.

As the methionine-requiring mutant will grow in the absence of methionine if plasma proteins are present, a protein-free extract of the plasma must be used for assay. Dilutions of this extract are made in fluid synthetic medium, a small inoculum of washed bacteria is added to each, and the tubes are incubated at 37° C. At the same time, a set of standard tubes containing known concentrations of methionine are similarly set up and incubated. After incubation, the turbidity of each culture is compared with the standard series in a photoelectric nephelometer.

METHOD

1. *Strains of Bacterium coli Employed.*—The L-methionine-requiring strain of *Bact. coli* K-12, mutant 58/161, was principally used in this work. It was found, however, that its requirement for methionine could be fully satisfied by either homocysteine or cystathionine,

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which constitute prior stages in methionine synthesis so that the biochemical block in this strain arises before the cystathionine stage. The following amino acids and growth factors were investigated to see whether they could support growth of this strain in the absence of methionine: cysteine, threonine, alanine, proline, aspartic acid, glycine, tryptophane, norleucine, glutamic acid, isoleucine, vitamin B_{12}, choline, calcium pantothenate, para-aminobenzoic acid, and adenine.

None of these substances was found to be capable of replacing methionine in supporting growth. Recovery and dilution experiments strongly suggested that the methionine precursors cystathionine and homocysteine were not present in biologic fluids in significant amounts. This was confirmed by repeating some of the assays with another mutant strain of *Bact. coli* K-12 (J^{5-3}) which requires both methionine and proline. Work on genetic recombination (Clowes and Rowley[3]) had suggested that in this strain the mutation to methionine-dependence had occurred at a different genetic locus from that of mutant 58/161. This suggestion was supported by the finding that homocysteine and cystathionine could not substitute for methionine in promoting growth of this mutant, nor could cysteine, vitamin B_{12}, aspartic acid, or adenine. Results of assays with the two strains were very similar, thus confirming that homocysteine and cystathionine are not normally present in the plasma in amounts which could interfere with the assay of methionine by mutant 58/161 of *Bact. coli* K-12.

2. *Preparation of Inoculum.*—An overnight broth culture of the assay strain was washed three times in 0.85 per cent sodium chloride and resuspended to the original volume in saline. One drop of this suspension (0.02 ml.) was used to inoculate the 10 ml. of medium in each assay tube.

3. *Medium.*—The medium employed had the following constitution:

Na_2 H PO_4	7 Gm.
K H_2 PO_4	3 Gm.
Mg. SO_4 (10 per cent soln.)	0.1 ml.
N H_4 Cl	1 Gm.
Dist. water to	1,000 ml.

The pH was adjusted to 7.2 and the medium sterilized by autoclaving. Before use, 1.25 ml. of sterile 20 per cent glucose solution was added to each 100 ml., giving a final glucose concentration of 0.25 per cent in the medium.

For assays employing strain J^{5-3}, 20 mg. of L-proline was added to each 1,000 ml. of the medium.

4. *Preparation of a Standard Curve.*—Tubes were prepared containing 10, 5, 4, 3, 2, 1 and 0.5 μg per milliliter of L-methionine in 10 ml. medium. After inoculation with the test organism, the tubes were incubated at 37° C. for forty-eight hours and the turbidity of each tube measured with a photoelectric nephelometer.* The turbidity in arbitrary units was then plotted against the concentration of L-methionine in μg per milliliter. Between 0.5 μg per milliliter and 5 μg per milliliter there is virtually a linear relationship (see Fig. 1).

5. *Deproteinization of Plasma.*[4]—Eleven and two-tenths milliliters of distilled water was added to 8 ml. of plasma in a bottle. This was then stoppered, shaken, and 2.8 ml. of 0.6 N H_2 SO_4 added slowly with mixing, followed by 2 ml. of 10 per cent sodium tungstate. The plasma was therefore diluted 1 in 3 in the preparation of a protein-free supernatant.

The bottle was then shaken for two to three minutes and centrifuged. The protein-free supernatant was pipetted off and used for the methionine assay after adjustment of the pH to 7.2 and sterilization by boiling for two minutes.

6. *Preparation of Assay Tubes.*—As the turbidity is proportional to the methionine concentration only over a limited range, several different dilutions of the supernatant were

*An "EEL" photoelectric nephelometer (made by Evans Electroselenium Ltd., Harlow, Essex, England) was used which measures, on an arbitrary scale, the amount of light reflected by the bacteria in the suspension. In order to obtain comparable readings, the instrument was adjusted each day so that a turbidity standard (known to be equivalent to 3.6 mg. of protein nitrogen of *Bact. coli* K-12) read 100 arbitrary units.

usually made in the basal medium. With low concentrations of methionine, such as are found in the fasting state, 5 ml. of plasma supernatant was added to 1 ml. of ten times concentrated synthetic medium and the total volume made up to 10 ml. with distilled water. Thus, the final dilution of the supernatant in the medium was 1 in 2. With higher methionine concentrations, 1 ml. or 0.5 ml. of plasma supernatant was added to 9 or 9.5 ml. of the synthetic medium, giving a dilution of the supernatant in the medium of 1 in 10 or 1 in 20.

7. *Calculation of Plasma Methionine Concentration.*—From the standard curve, the methionine concentration in micrograms per milliliters in one of the dilutions of the supernatant set up was determined. This figure was then multiplied by the dilution factor of the supernatant in the medium of the particular tube used, and also by three, which is the dilution of the original plasma in the protein-free supernatant.

Thus: Methionine concentration in μg per milliliter of the original plasma = Methionine concentration in the test tube read off the standard curve × Dilution of supernatant in the medium × 3

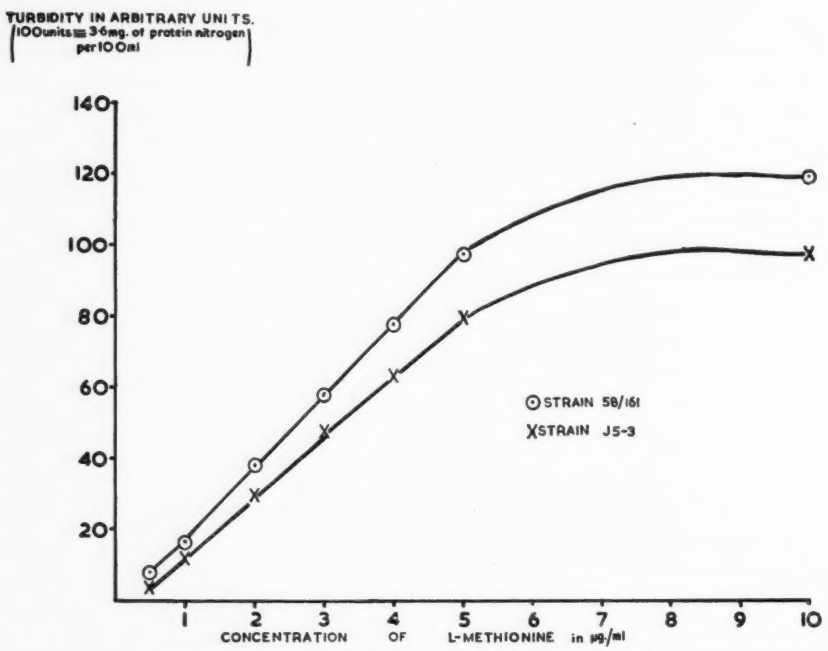

Fig. 1.—Standard curve of growth in arbitrary turbidimetric units as related to concentration of L-methionine in medium.

RESULTS

1. *Recovery Experiment.*—The L-methionine in normal plasma and in the same sample of plasma to which 100 μg per milliliter of L-methionine had previously been added was estimated.

	NEPHELOMETER READINGS IN ARBITRARY UNITS		ESTIMATED CONCENTRATION OF METHIONINE IN MICROGRAMS PER MILLILITER
	DILUTIONS OF SUPERNATANT IN MEDIUM		
	1:2	1:20	
Plasma	20	–	6
Plasma methionine	–	34	108

2. *L-methionine Levels in Patients With Cirrhosis of the Liver.*—Ten grams of DL-methionine was given daily for two to six days to 9 patients with cirrhosis of the liver. The L-methionine morning plasma levels varied from 9 to 69 μg per milliliter before the administration of methionine. They rose to 20 μg per milliliter, or above, in every patient receiving this amino acid. In one case, after six days a level of 375 μg per milliliter was reached.

In a series of 10 parallel estimations of a single specimen, the average reading was 43.1 arbitrary units and the standard deviation ± 2.

DISCUSSION

Although quantitative chromatographic methods for the estimation of amino acids in biologic fluids have recently been developed (for example, column chromatography by Moore and Stein,[5] and paper chromatography by Smith and Tompsett[6]), microbiologic methods probably remain more specific and sensitive than chemical estimations (Wheeler and Gyorgy[7]). The usual microbiologic methods employ *Streptococcus faecalis* (Stokes and co-workers[8]) or *Leuconostoc mesenteroides* P_{60} for L-methionine and *Lactobacillus fermentans* 36 for DL-methionine (Kinsell and associates[9]). All these organisms require complex synthetic media for assay.

The *Bact. coli* mutants used in this method require L-methionine for growth. Except for cystathionine and homocysteine, which can replace this amino acid as an essential growth factor for strain 58/161, none of the amino acids, B vitamins, and growth factors tested could replace methionine in promoting growth. Both strains gave very similar results, and it is concluded that cystathionine and homocysteine are not present in the plasma in amounts sufficient to invalidate this assay method.

The medium used in this method is much simpler than that required by the other test organisms. There appears to be no reason why other amino acids and B vitamins should not be assayed by a similar method using *Bact. coli* mutants.

The results obtained by this method are very similar to those of Kinsell and associates[2] who administered 9 Gm. of DL-methionine daily, as compared to 10 Gm. in the present series. Using *Leuconostoc mesenteroides* P_{60}, they found that elevation of the fasting level to above 20 μg per milliliter occurred in all patients with liver disease, but not in normal subjects. The present investigation thus confirms that methionine utilization is impaired in patients with cirrhosis of the liver.

SUMMARY

A microbiologic method for the assay of L-methionine in plasma, using a methionine-requiring *Bact. coli* mutant, is described. The medium used is much simpler than that required by other test organisms. The method has been applied to the investigation of the abnormal methionine metabolism in patients with cirrhosis of the liver.

It is suggested that other amino acids and B vitamins might be assayed using *Bact. coli* mutants.

I wish to thank Dr. Sheila Sherlock for stimulating my interest in methionine metabolism, Lord Stamp for his encouragement, Dr. W. Hayes for much help and advice and also for strain 58/161, and Dr. C. E. Dalgliesh for valuable suggestions.

I am also grateful to Dr. R. C. Clowes of the Wright Fleming Institute, St. Mary's Hospital, London, for strain J^{5-3} and Dr. D. D. Woods, F.R.S., of the Biochemistry Department, Oxford University, for a gift of cystathionine.

REFERENCES

1. Himsworth, H. P.: Lectures on the Liver and Its Diseases. Comprising the Lowell Lectures delivered at Boston, Mass., in March, 1947, Oxford, England, Blackwell Scientific Publications.
2. Kinsell, L. W., Harper, H. A., Giese, G. K., Magan, S., McCally, D. P., and Hess, J. R.: Studies in Methionine Metabolism; Fasting Plasma Methionine Levels in Normal and Hepatopathic Individuals in Response to Daily Methionine Ingestion, J. Clin. Invest. **28**: 1439, 1949.
3. Clowes, R. C., and Rowley, D.: Some Observations on Linkage Effects in Genetic Recombination in Escherichia Coli K-12, J. Gen. Microbiol. **11**: 250, 1954.
4. Hier, S. W., and Bergeim, O.: Microbiological Determination of Free Leucine, Isoleucine, Valine, and Threonine in Dog Plasma, J. Biol. Chem. **161**: 717, 1945.
5. Moore, S., and Stein, W. H.: Chromatography of Amino Acids on Sulfonated Polystyrene Resins, J. Biol. Chem. **192**: 663, 1951.
6. Smith, D. C., and Tompsett, S. L.: The Quantitative Determination of Amino-acids in Urine Used in Conjunction With One-dimensional Paper Chromatography, J. Clin. Path. **7**: 79, 1954.
7. Wheeler, J. E., and Gyorgy, P.: Studies of Urinary Excretion of Methionine by Normals and by Patients Having Liver Disease, Am. J. M. Sc. **215**: 267, 1948.
8. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C.: Microbiological Methods for the Determination of Amino Acids, J. Biol. Chem. **160**: 35, 1945.
9. Kinsell, L. W., Harper, H. A., Barton, H. C., Michaels, S. D., and Weiss, H. A.: Rate of Disappearance From Plasma of Intravenously Administered Methionine in Patients With Liver Damage, Science **106**: 589, 1947.

AN ARTIFACT IN THYROXINE CHROMATOGRAPHY USING DIOXANE

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TAUROG and associates[1] found an unexpectedly large iodide^{131} component when they subjected radioiodinated thyroxine to chromatography with collidine and water (in a ratio of 100:35.5) in an atmosphere of ammonia. Stanley[2] noted deterioration of iodinated tyrosines when heated to 105° C. with 2N sodium hydroxide. We wish to report the degradation of thyroxine that has been exposed to dioxane which has not been freed of its peroxide impurities.

MATERIALS AND METHODS

Chromatography.—The ascending paper technique of Gross and co-workers[3] was used, employing a mixture of n-butanol and dioxane (4:1) equilibrated with five parts of 2N ammonium hydroxide. The dried strips of paper were scanned by a Geiger-Müller mica-window tube, with automatic recording of the radioactivity. Certain strips were taped to "no-screen" (Kodak) x-ray film for ten days; the film was then developed for the identification of zones of radioactivity.

Column chromatography employing kieselguhr, based on the method of Gross and Pitt-Rivers[4] as modified by Braasch and co-workers[5] was used also. It consists of (a) the supernate from a mixture of n-butanol, chloroform, and 0.5N sodium hydroxide (4:1:5) as a solvent for thyroxine, triiodothyronine, and iodide, and (b) the supernate from a mixture of n-butanol, n-propanol and 0.5N sodium hydroxide (9:1:1) as a solvent for the tyrosines. The effluent from the column was carried through a small-bore tube across the end of a sodium iodide (thallium-activated) scintillation crystal; the counting of gamma rays so detected was recorded on calibrated paper so that areas representing the integrated radioactivity could be determined by planimetry.

Dioxane.—Stock bottles of 1,4-dioxane were found to contain variable amounts of peroxide: namely, 0.09, 0.12, and 0.20 molar, respectively, from a newly purchased bottle, one a few years old, and one that was probably more than fifteen years old. A portion of the oldest supply was distilled to obtain both a peroxide-free dioxane and a concentrate of the impurity (1.17 M). We considered it unsafe to concentrate further because of the possibility of explosion. The oily concentrate released iodine from sodium iodide explosively; when mixed with thyroxine, it disorganized the molecule into several chromatographically unrecognizable parts.

Radiothyroxine.—I^{131}-labeled thyroxine, dissolved in 50 per cent propylene glycol, was used in these studies.*

RESULTS

Stock dioxane was used in our early attempts to purify commercial radiothyroxine by paper chromatography. The solution of thyroxine was applied

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*Obtained from Abbott Laboratories, North Chicago, Ill.; its use was authorized by the Atomic Energy Commission.

to strips of Whatman No. 1 paper, dried and chromatographed with the butanol-dioxane-ammonia solvent. The region to which thyroxine migrated was eluted with a small amount of n-butanol and rechromatographed. However, iodide, as well as other radioactive components, appeared on the second strip of paper. Further chromatographic "purification" continued to yield small amounts of radioiodide. When this procedure was repeated with peroxide-free dioxane, the thyroxine appeared as the only component on the chromatogram.

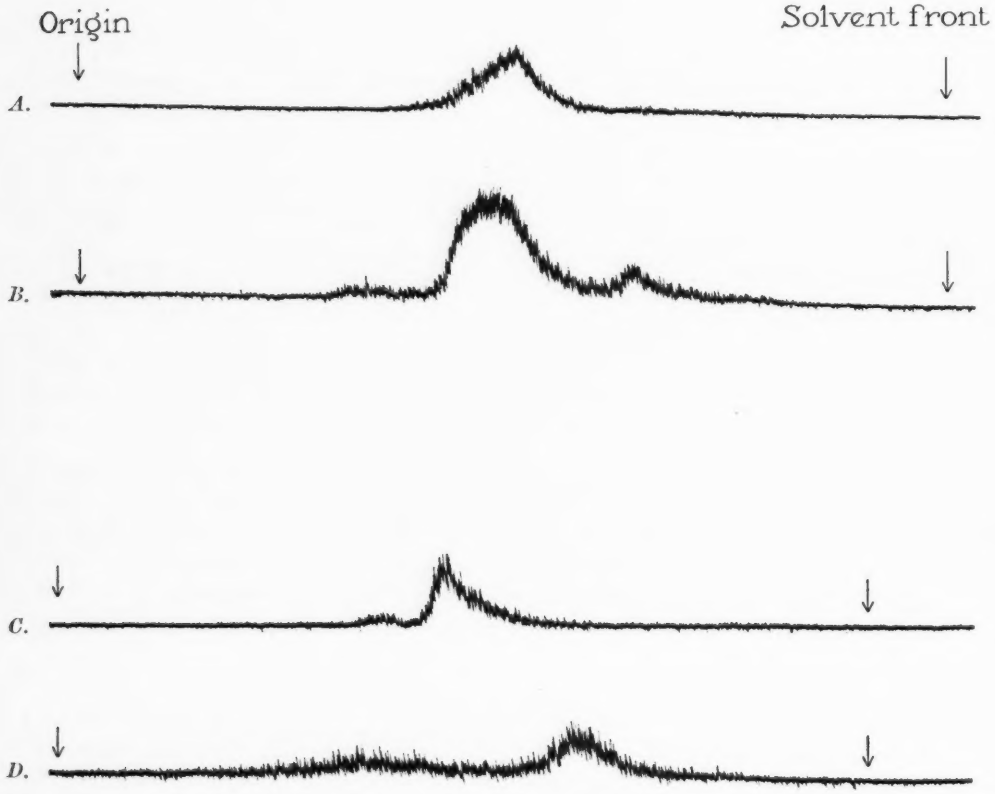

Fig. 1.—Tracings of the radioactivity on paper chromatograms. I^{131}-labeled L-thyroxine was applied at the solvent front of all four papers; *A* and *B* were chromatographed for 8½ hours with butanol-dioxane-ammonia, *C* and *D* for 16 hours. The dioxane in the solvent used for strips *A* and *C* was freed of peroxides by distillation, whereas that for *B* contained 0.09 M and for *D* contained 0.20 M.

Fig. 1 illustrates typical results. *A* and *C* show the distribution following chromatography for periods of eight and one-half and sixteen hours, respectively, of radiothyroxine in the presence of purified dioxane; *B* and *D* show the distribution at the same intervals of time when impure specimens of dioxanes were used. The zone of thyroxine in B is still prominent but smaller components have appeared on either side; they have the mobility of triiodothyronine (faster moving) and iodide. In *D*, the thyroxine is seen to have almost disappeared and the zones of triiodothyronine and iodide are predominant. In other chromatograms, no clear division of degraded products was seen; it appeared as if con-

tinuous breakdowns were occurring during the chromatographic process. Similar patterns were obtained with commercial I^{131}-triiodothyronine, which gave a continued appearance of iodide as well as slower-moving components (tyrosines?).

Fig. 2 compares autoradiograms of paper chromatographic strips; butanol extracts of serum from a patient who had received a therapeutic dose of radioiodine for the treatment of a malignant lesion of the thyroid were used. Fig. 2, *A* is a reproduction of x-ray film exposed to the paper that was chromatographed with purified dioxane; it shows a large zone of thyroxine, a fainter zone of iodide and an unidentified spot at the solvent front. In *B*, with impure dioxane in the solvent, a series of unidentified opacities resulted.

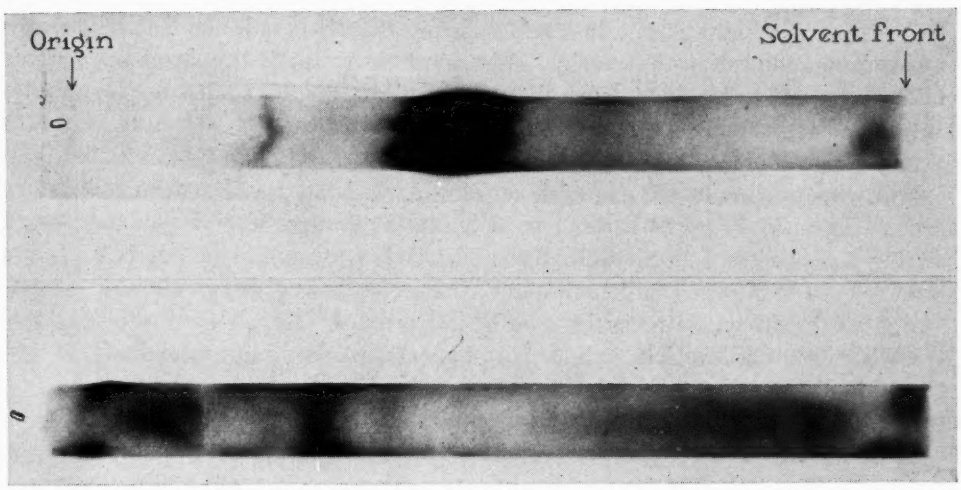

Fig. 2.—Autoradiographs of radioactivity on paper chromatograms. Acid-butanol extracts of serum were applied to both papers; the dioxane portion of the solvent for *A* was free of peroxide, whereas that for *B* contained 0.20 M peroxide. The serum was obtained from a patient with thyroidal carcinoma 3 days after a therapeutic dose of radioiodine.

TABLE I. PER CENT OF TOTAL RADIOACTIVITY IN EACH FRACTION DETERMINED BY COLUMN CHROMATOGRAPHY (KIESELGUHR) OF I^{131}-LABELED THYROXINE TREATED WITH DIOXANE CONTAINING VARYING AMOUNTS OF PEROXIDES

FRACTION IN EFFLUENT FROM COLUMN*	DIOXANE, AMOUNT OF PEROXIDES		
	NONE	0.09 M	0.20 M
Thyroxine	92.1	85.2	67.6
Triiodothyronine	1.3	4.0	5.5
Iodide	3.5	6.9	2.7
Solvent change†	2.4	2.6	3.4
Diiodotyrosine	0.5	0.6	8.3
Monoiodotyrosine	0.2	0.2	7.7
Residue‡	0.0	0.5	4.8

*The components in the effluent were not identified but agreed with the mobility of known compounds added to comparable columns.

†This is a radioactive component appearing in the effluent when the solvent was changed from butanol-chloroform to butanol-propanol.

‡This represents the residual radioactivity of the column as flushed out with water.

Table I lists the radioactive components derived from I^{131}-labeled thyroxine that was mixed for several hours with dioxane containing various concentrations of peroxides. Chromatography was through a column of kieselguhr. The com-

ponents are presumptive in that their mobilities agree with those of known compounds run through such a column, but they may not actually be the presumed ones, since use of a mixture of carrier-free iodide^{131} with dioxane impurities also led to the appearance of several components in the column; these could hardly have been thyronines or tyrosines. If certain of the degradation products of thyroxine are iodinated tyrosines, labeled at the 3 or 5 position, one must postulate an I^{127}-I^{131} exchange, since the thyroxine was labeled at only the 3′ or 5′ position.

COMMENT

The finding that peroxide impurities in dioxane can degrade thyroxine and triiodothyronine is not surprising since these peroxides have strong oxidative properties. However, a lack of awareness of this possibility may lead to the unjustified assumption that various substances that are not thyroxine are present in a solution of thyroxine. Even dioxane freed of peroxides must be viewed with suspicion. In one instance, peroxides developed in such a preparation to the extent of 0.014 M during a year's time, although the material was kept frozen except when melted briefly for each actual use. Further, even with pure dioxane, chromatography requires a number of hours, and some degradation may occur during the process. As a result, the appearance of traces of triiodothyronine in the presence of a large component of thyroxine may not be valid.

A study was made recently of radioactive iodine in the serum of 50 patients treated with radioactive iodine for hyperthyroidism or carcinoma of the thyroid. When butanol extracts of their serums were chromatographed on paper, small amounts of radioactivity practically always were present in the zone ascribed to triiodothyronine; in no case was this radioactivity enough to quantitate with any precision. In the light of a potential artifact with the dioxane solvent, we cannot be sure that triiodothyronine actually was present in more than traces in any instance.

SUMMARY

Thyroxine and triiodothyronine are readily degraded by the peroxides that tend to develop spontaneously in 1,4-dioxane. The resulting artifacts may be misleading in quantitative chromatographic analyses of thyroxine-containing solutions, such as serum, when dioxane is one constituent of the solvent.

REFERENCES

1. Taurog, A., Briggs, F. N., and Chaikoff, I. L.: I^{131}-labelled L-thyroxine. II. Nature of the Excretion Product in Bile, J. Biol. Chem. **194:** 655-668, 1952.
2. Stanley, P. G.: Artefacts Produced From 3-Iodo-tyrosine and 3:5-Di-iodo-tyrosine by Heating With Alkali, Nature **171:** 933-934, 1953.
3. Gross, J., Leblond, C. P., Franklin, A. E., and Quastel, J. H.: Presence of Iodinated Amino Acids in Unhydrolyzed Thyroid and Plasma, Science **111:** 605-608, 1950.
4. Gross, J., and Pitt-Rivers, R.: The Identification of 3:5:3′-L-triiodothyronine in Human Plasma, Lancet **1:** 439-441, 1952.
5. Braasch, J. W., Flock, E. V., and Albert, A.: A Quantitative Method for the Separation of the Iodinated Amino Acids of Thyroid Tissue, Endocrinology **55:** 768-776, 1954.

A METHOD FOR PROTEIN-BOUND IODINE: THE KINETICS AND THE USE OF CONTROLS IN THE ASHING TECHNIQUE

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IN RECENT years the estimation of serum protein-bound iodine (PBI) has become an important adjunct to the tools available to the diagnostician in confirming the existence of thyroid dysfunction. The PBI determination offers a more reliable and reproducible indication of thyroid function than the basal metabolic rate, which can be markedly affected by environmental and physiologic factors.

Current methods for the determination of serum PBI have been deemed adequate. Nevertheless, in a control study conducted in this laboratory by Benenson, Thompson, and Klugerman,[1] an estimated error of approximately 20 per cent was obtained by the ashing technique. This large error precluded the use of PBI determinations as a means of detecting borderline cases of thyroid pathology or for the purpose of therapeutic control. The requirements of an adequate method must not only include sensitivity to microquantities of iodide but also be capable of an accuracy, reproducibility, and a simplicity which would enable a technician with minimal technical ability to arrive at consistent results. With this in mind, the procedure at this laboratory was subjected to intensive scrutiny.

Several different methods have been suggested for destroying the organic matter in order to set free the bound iodide. There are three techniques for protein digestion and preparation of the sample for iodide analysis which have found wide application in clinical laboratories: the oxidation-distillation of Chaney,[2] the wet-digestion of Zak, Willard, Myers, and Boyle,[3] and the dry-ashing method of Barker, Humphrey, and Soley.[4] Van Zyl[5] and Moran[6] have carefully studied the oxidation-distillation method, and O'Neal and Simms[7] have evaluated the wet-digestion method. No similar effort has yet been made to study and possibly improve the dry-ashing sodium carbonate method. This technique has certain inherent advantages over that of the wet-digestion process: less attention is required during digestion, there is less likelihood of encountering explosive mixtures, there is absence of such interfering oxidants as chromates, and smaller samples are required for processing. Moreover, Zieve, Dahle, and Schultz[8] have reported that there is more discriminative effectiveness in borderline cases with the ashing technique.

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The colorimetric technique sensitive to trace quantities of iodide was first described by Sandell and Kolthoff,[9] who studied the catalytic action of iodide on the ceric-arsenite system:

$$2\ Ce^{4+} + As^{3+} \xrightarrow{I^-} 2\ Ce^{3+} + As^{5+}$$

By measuring the time required for the complete decolorization of the ceric ion, they were able to show a linear relationship between reaction time and iodide concentration. Current methods found in the literature utilize this concept of the decolorization of ceric ion as effected by the iodide present but differ in the manner of estimating the concentration of iodide from the data obtained. With the advent of instrumental analysis, Salter and McKay,[10] and Barker and associates[4] plotted log O.D. versus time, and Caraway[11] based a series of calculations upon the equation of a first-order reaction.

It is our intent to demonstrate, by means of kinetic data, that the calculations based upon the plot of log O.D. versus time or upon the equation of a first-order reaction are not quite valid because the concentration of ceric ion is not directly proportional to absorbance on instruments (other than the Beckman DU) commonly used in the laboratory.

A modification of Barker's dry-ashing method for PBI will be presented in an effort to simplify, by standardization, a reliable test for evaluating the extent of thyroid dysfunction. The effects of variable ionic constituents on the catalytic system and methods of control to insure the maximum of accuracy and reproducibility will also be described.

Section I

Materials and Methods

The sample should be collected in chemically clean tubes. The use of tincture of iodine, or any form of iodine, when drawing blood from a patient is a source of error. Impurities in certain reagents may so alter the catalytic reaction that the range of linearity is adversely affected. Miscellaneous catalysts, such as osmium, accelerate the reaction rate; inhibitors such as fluorides, cyanides, and silver slow the reaction rate. Therefore, all reagents should be of highest purity obtainable. The reagents should be tested and so selected that the blank obtained is extremely low. Distilled water should be redistilled, in an all-glass distillation apparatus, from alkali (a pellet or two of NaOH or KOH) for use in the procedure. Glassware should be washed in hot water containing any good detergent and carefully rinsed with tap water and then redistilled water. Pipettes should be cleaned in acid dichromate solution.

As an added precaution, a separate room for performing PBI determinations is highly recommended to preclude chance contamination from iodine fumes or mercury vapor which may originate from other sections of the laboratory.

I. Reagents.—

Zinc sulfate: Dissolve 100 Gm. of $ZnSO_4 \cdot 7H_2O$ in water and dilute to a liter in a volumetric flask.

Sodium hydroxide—(0.5 N): Dissolve 20 Gm. of sodium hydroxide in water and dilute to a liter mark in a volumetric flask. The zinc sulfate and sodium hydroxide should be so related that when 10.0 ml. of zinc sulfate is diluted with 50 to 70 ml. of water and titrated with the sodium hydroxide, then 10.8 to 11.2 ml. of the 0.5 N NaOH is required to produce a faint permanent pink color with phenolphthalein.

Hydrochloric acid (2 N): Dilute with water 170 ml. C.P. concentrated hydrochloric acid to a liter in a volumetric flask. Titrate against standard 1.0 N sodium hydroxide.

Sulfuric acid (7 N): Dilute 200 ml. of concentrated sulfuric acid with water to one liter in a volumetric flask.

Sodium carbonate (4.0 N or 2.0 M): Dissolve 212 Gm. of Na_2CO_3 C.P. in water and dilute to one liter in a volumetric flask. Titrate against standard 1.0 N hydrochloric acid using a methyl orange indicator.

Ceric sulfate (0.012 N or 0.012 M): Dissolve 8.02 Gm. of ceric ammonium sulfate, $Ce(SO_4)_2\ 2(NH_4)_2 \cdot SO_4 \cdot 4H_2O$, in a liter flask containing 500 ml. of water and 230 ml. 7 N H_2SO_4. When the solution is clear, dilute with water to the liter mark.

Estimation of workable concentration of ceric ion: Prepare a 0.0012 M solution of ceric sulfate by diluting 5 ml. of the 0.012 M to 50 ml., in a volumetric flask, with 1.68 N H_2SO_4 (23 ml. concentrated H_2SO_4 diluted with water to 100 ml.). Add the indicated amounts of the 0.012 M and 0.0012 M ceric sulfate solutions to each of eleven cuvettes; then add the indicated amounts of 1.68 N H_2SO_4 and 8.0 ml. of water for the following dilutions of ceric ion (total volume, 12 ml.):

MILLILITERS CERIC SULFATE	MILLILITERS 1.68 N H_2SO_4	MILLIMOLES CERIC ION/LITER	2 + LOG $[Ce^{4+}]$* MILLIMOLES/LITER
1. 4.0 ml. 0.012 M	0	4.0	2.602
2. 3.0 ml. 0.012 M	1.0	3.0	2.477
3. 2.5 ml. 0.012 M	1.5	2.5	2.398
4. 2.0 ml. 0.012 M	2.0	2.0	2.301
5. 1.5 ml. 0.012 M	2.5	1.5	2.176
6. 1.0 ml. 0.012 M	3.0	1.0	2.000
7. 0.5 ml. 0.012 M	3.5	0.5	1.699
8. 4.0 ml. 0.0012 M	0	0.4	1.602
9. 3.0 ml. 0.0012 M	1.0	0.3	1.477
10. 2.0 ml. 0.0012 M	2.0	0.2	1.301
11. 0.5 ml. 0.0012 M	3.5	0.05	0.699

*[ ] = ion concentration.

Read per cent transmittance at 420 mμ against a water blank set at 100 per cent transmittance. Plot 2 + log $[Ce^{4+}]$ versus per cent transmittance on standard graph paper, as shown in Fig. 1. The value of 2 + log $[Ce^{4+}]$, which corresponds to the lowest transmittancy value on the linear portion of the curve, is selected as the optimum ceric ion concentration for the particular instrument. For example, with the use of the Evelyn colorimeter, Fig. 1 is made to show that 2 + log $[Ce^{4+}]$ of 2.27, corresponding to 22 per cent transmittance, is the optimum ceric ion concentration. The antilog of 0.27 is equal to 1.85 mM of ceric ion. Millimoles of ceric ion multiplied by 4.346 equals grams of ceric ammonium sulfate to be weighed out and dissolved in 230 ml. of 7 N H_2SO_4 to prepare a liter of solution; or 1.85 × 4.346 equals 8.02 Gm.

Sodium arsenite (0.12 N): In a liter flask, dissolve 5.84 Gm. of As_2O_3 in about 30 ml. of 4 per cent NaOH, warming to hasten solution. Add about 300 ml. of water and sufficient dilute H_2SO_4 to make the solution slightly acid to litmus. (If the 7 N H_2SO_4 is used, about 4.5 ml. will be required.) The solution is diluted with water to the liter mark. If too high a blank is obtained with this reagent, purify the As_2O_3 by sublimation. This can be done by heating the As_2O_3 in a porcelain dish, covered with a watch glass or petri dish, over a free flame. The sublimed As_2O_3 condenses on the sides of the dish. If sodium arsenite is used, dissolve 7.80 Gm. of $NaAsO_2$ in distilled water and dilute to 1 liter. No acid or alkali is required with this salt.

Iodide standard: Either NaI or KI of highest purity may be used. Keep dry in desiccator.

Dissolve 118.1 mg. of NaI or 130.8 mg. of KI in water and dilute with distilled water to 1 liter in a volumetric flask. (1 ml. = 100.0 μg I^-).

Dilute Stock is obtained by diluting 4 ml. of the above-mentioned solution to 1 liter (1 ml. = 0.40 μg I^-) with distilled water.

To prepare a working standard, dilute 10 ml. of dilute stock to 100 ml. (1 ml. = 0.04 μg I^-) with distilled water.

All of these solutions have been found to keep indefinitely at refrigerator temperatures. It is preferable, however, to prepare a new working standard every week or two.

Apparatus:

1. 15 × 125 mm. Pyrex test tubes.
2. Muffle furnace.
3. Oven set at 85° to 95° C.
4. Photoelectric colorimeter.

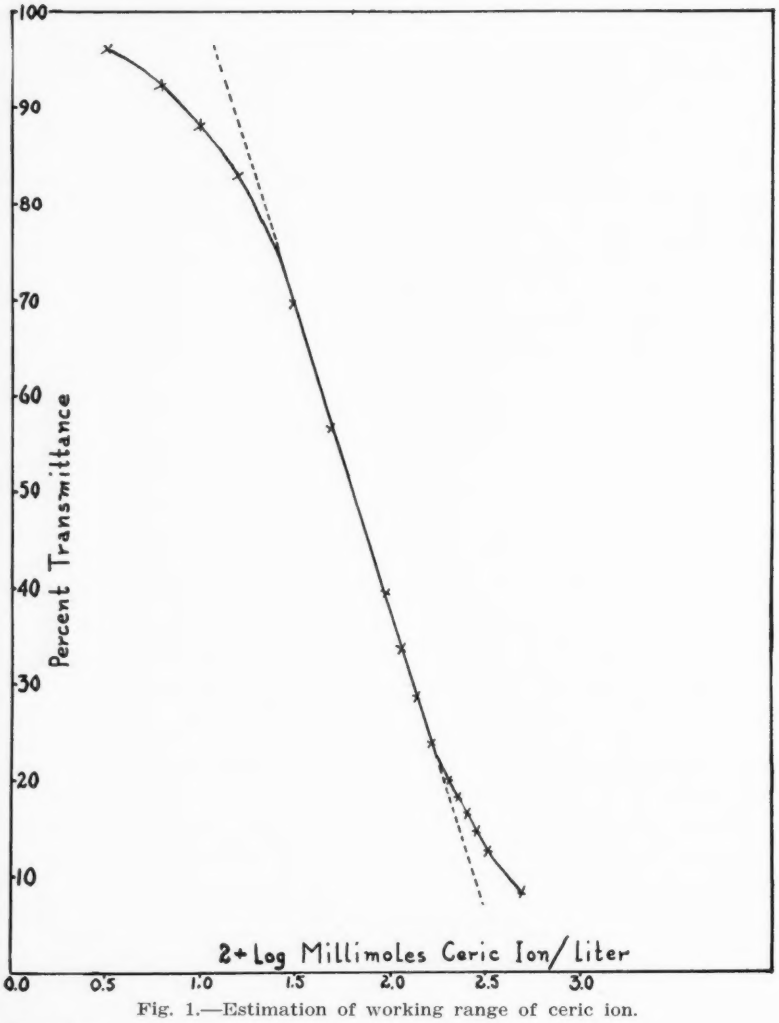

Fig. 1.—Estimation of working range of ceric ion.

II. Procedure.—

Precipitation and washing of protein:

1. Pipette 1 ml. of serum or plasma into a 15 × 125 mm. Pyrex test tube (in triplicate).

2. Add 7.0 ml. of water. Prepare a reagent blank in quadruplicate (two of these tubes will be used subsequently to prepare a standard in duplicate), using 8 ml. of water in a similar tube.

3. To all tubes, add 1.0 ml. of 10 per cent zinc sulfate solution. Mix with a stirring rod about 2 mm. in diameter, using a different stirring rod for each tube.

4. Add 1.0 ml. of 0.5 N NaOH solution. Mix thoroughly to insure even distribution of alkaline zinc sulfate and complete precipitation of proteins. Remove adhering material from rod by rubbing it with a rotary motion against inside wall of tube, and lay rod aside for subsequent washings.

5. Centrifuge for ten minutes at about 3,500 r.p.m.

6. Pour off and discard supernatant fluid.

7. Add 8 ml. of water to each tube, mix thoroughly with same stirring rod to break up clumps and distribute the proteins well. (Avoid too vigorous mixing, as this will cause incomplete separation of the zinc proteinate during centrifugation).

8. Centrifuge and discard supernatant.

9. Repeat washing twice more. After the last decantation of supernatant fluid, allow tube to drain over filter paper.

10. Add 1.0 ml. of 4.0 N sodium carbonate solution and thoroughly stir the precipitate with the same stirring rod used in the washings. Remove adhering material on stirring rod by rubbing it against inside wall of tube and wash off remaining material into tube with about 0.2 ml. distilled water. Discard stirring rods.

Drying and incineration:

1. Roll tube between fingers in almost a horizontal position to distribute the precipitate evenly along the wall about one-half the way up along the length of the tube.

2. Place tubes in an oven set at 85-95° C. to drive off the water. Tubes are placed in a test tube rack inclined at about 45° angle. This is usually carried out overnight.

3. After thorough drying of precipitate, place tubes at an angle of about 30° in a muffle furnace. Raise the temperature slowly at first to prevent ebullition of material. Ashing is carried out for 2 to 3 hours at 1,112° ± 45° F. (600 ± 25° C.), or until most of the carbon has been oxidized. At higher temperatures, loss of iodide by volatilization (M.P. 651° C.) may occur.

4. Remove tubes and cool to room temperature, in a covered preserving jar to prevent contamination from air.

Colorimetric determination:

1. Neutralize the alkaline salts by adding rapidly 2 ml. of 2 N HCl to drive off the H_2S and CO_2 by the consequent effervescense. (No loss of material was experienced during this step).

2. Perform each sample in triplicate and a reagent blank in duplicate. Add 1.0 ml. of 7 N H_2SO_4 and 3.0 ml. of water to two of the sample tubes. To the third tube of sample may be added 1.0 ml. 7 N H_2SO_4, 2.0 ml. water, and 1.0 ml. of working standard (.004 μg I^-). (This internal standard serves a dual purpose: to detect the possible presence of an inhibitory substance in the sample, and to check accuracy of the colorimetric procedure by recovery of the added iodide.) The standard (in duplicate) is prepared by adding to the third and fourth tubes containing reagent blank 1.0 ml. 7 N H_2SO_4, 2.0 ml. water, and 1.0 ml. working standard.

3. If the tubes contain insoluble matter, centrifuge briefly and use supernatant for remainder of test. Pipette 5 ml. of each mixture into colorimeter tubes.

4. Add 0.5 ml. of arsenious acid solution to each colorimeter tube. (If the H_2S is not driven off completely during the acidification of the ashed sample, yellow arsenic trisulfide may precipitate out at this point ($2\ As^{3+} + 3\ H_2S \longrightarrow As_2S_3 + 6H^+$). It is not necessary to allow the solutions to equilibrate, but continue with the addition of ceric sulfate reagent as soon as possible. The mixture is kept at room temperature (about 25° C.) for the remainder of the procedure.

5. Add exactly 1.0 ml. of ceric sulfate reagent from a burette and take the initial reading immediately. Since the actual determination of iodide is accomplished by measuring iodide catalysis of the rate of decolorization of the yellow ceric sulfate by arsenious acid, the times at which the colorimeter readings are made must be set up. One milliliter of the ceric sulfate reagent must be added to each tube with adequate time allowance for reading the color change in the previous tube. This is accomplished by leaving a 30-

second interval between tubes. Colorimeter readings are made at 0, 6, and 12 minutes after addition of the ceric sulfate reagent to each tube. Twelve samples can conveniently be run in each series.

6. Read per cent transmittance of all tubes at 420 mμ against a water blank set at 100 per cent transmittance.

If the Evelyn colorimeter is used and the 12-minute readings exceeds 75 per cent transmittance (upper limit of workable range of instrument), only the 6-minute reading is considered in the calculation and multiplied by 2 to obtain a calculated 12-minute reading.

III. Calculation.—

The average 12-minute reading for each tube may be determined by using the formula of Klugerman[11]:

$$\frac{2\,b + c - 3a}{2} = D$$

a = zero minute reading in per cent transmittance
b = 6-minute reading in per cent transmittance
c = 12-minute reading in per cent transmittance
D = average increase of per cent transmittance in the interval, 0 to 12 minutes.

If the workload is heavy and good linearity is obtained for varied concentrations of iodide, the number of tubes in a single series may be doubled by taking only the 0- and 12-minute readings and the △ per cent T in 12 minutes may be substituted for D in the calculation, except in those samples where the 12-minute reading will exceed the workable range of the instrument.)

$$\frac{D\text{ sample} - D\text{ blank}}{D\text{ standard} - D\text{ blank}} \times 4.0 = \text{micrograms per cent PBI in sample}$$

A calibration curve is prepared whenever new reagents are prepared, in order to check linearity in the range used. Into each of five tubes, 1.0 ml. 4 N sodium carbonate, 2.0 ml. 2 N HCl, 1.0 ml. 7 N sulfuric acid, and quantities of standard (equivalent to 0, 0.04, 0.08, 0.12 and 0.20 μg I^-) and water to bring total volume to 6.0 ml. Transfer five milliliter aliquots to colorimeter tubes and treat subsequently as are the unknown samples beginning with step 4 of the colorimetric procedure. With the blank subtracted, plot per cent T against concentration of iodide.

Control Sera: If practical, analyze three previously standardized control sera having concentrations of approximately 3.0, 5.0, and 8.0 μg per cent PBI per 100 ml., together with sample, blank, and standard. The preparation of such sera and a recommended method of quality control will be discussed in Section III.

Section II

Instrumentation, Kinetics, and Effects of Variable Ionic Constituents in the Catalytic Reaction

1. Instrumentation.—

a. The absorption characteristics of the yellow color of the ceric ion were determined on five photoelectric instruments: a spectrophotometer with fine resolution (Beckman DU), two ordinary spectrophotometers (Coleman Jr. 6 and 6A), and two filter photometers (Evelyn and Klett-Summerson). The absorbancy was plotted against concentration of ceric ion (Fig. 2) to determine the extent of their adherence to Beer's law. The Coleman Jr. 6 and 6A, the Evelyn, and the Klett-Summerson produce a parabolic type of curve. The linearity, or adherence to Beer's law, is approached only approximately over a rather limited range. The Coleman Jr. 6 shows the poorest sensitivity and the Coleman Jr. 6A, the best sensitivity. The Beckman DU has both excellent linearity and sensitivity, which would make it the most desirable instrument except that it is not practical for measuring reaction rates of several samples within a given time.

The three instruments immediately available to this laboratory were the Beckman DU, the Coleman Jr. 6, and the Evelyn colorimeter. Since the Evelyn possesses the most desirable operational features, this instrument was chosen to measure the catalytic system for PBI.

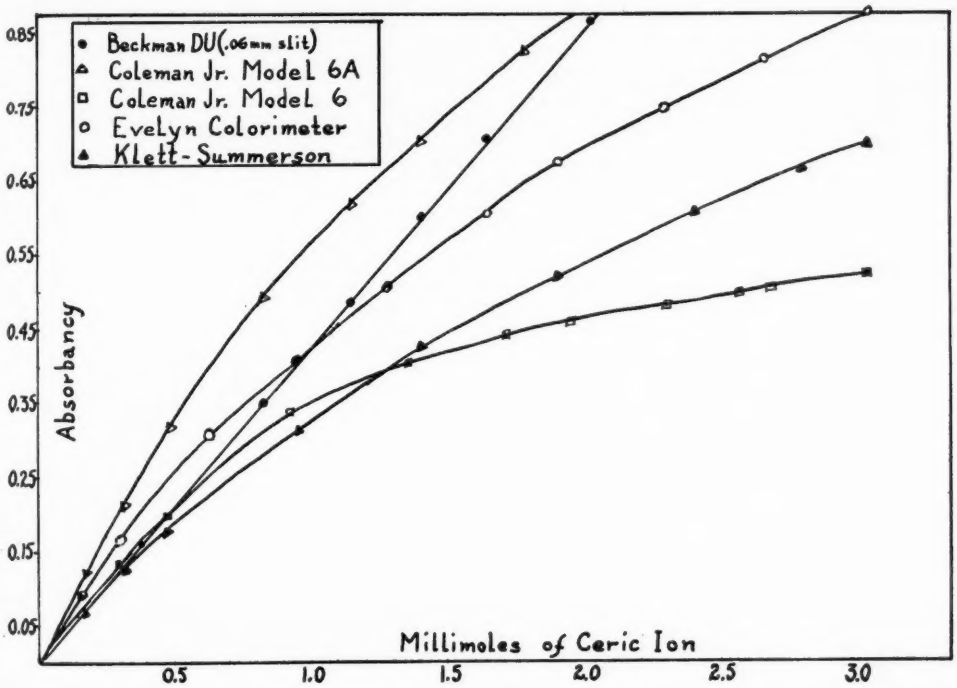

Fig. 2.—Comparison of various types of spectrophotometers and filter colorimeters (25° C.).

b. No particular advantage was found in using 440 mμ rather than 420 mμ, as suggested by some workers in their efforts to obtain better adherence to Beer's law. The use of 440 mμ results in increasing initial transmittance readings to the more accurate part of the galvanometer scale when the concentration of ceric ion originally proposed by Barker and associates is used. This arbitrary means of securing linearity may result in some loss of sensitivity. In the procedure described herein, the optimum working concentration of ceric ion was determined at 420 mμ.

2. Kinetics.—

If the rate-determining step in the decolorization of the ceric ion is assumed to be the reaction $Ce^{4+} \xrightarrow{I^-} Ce^{3+}$ and that the reaction $As^{3+} \xrightarrow{I^-} As^{5+}$ proceeds rapidly, then the following equation for a first-order reaction may describe the system:

$$(1)\quad 2.303 \log \frac{[Ce^{4+}]_o}{[Ce^{4+}]_t} = Kt$$

Substituting absorbance for concentration of ceric ion:

$$(2)\quad 2.303 \log \frac{O.D._o}{O.D._t} = Kt$$

It is apparent that if equation (2) is to be used as a basis of calculations, then log O.D. must be linear to time. From data obtained on the Beckman DU and the Evelyn colorimeter, log O.D. was plotted against time in Fig. 3. Since dilutions of ceric ion showed adherence to Beer's law on the Beckman DU, the linearity observed with this instrument confirms the first-orderliness of the catalytic reaction and the validity of equation (2).

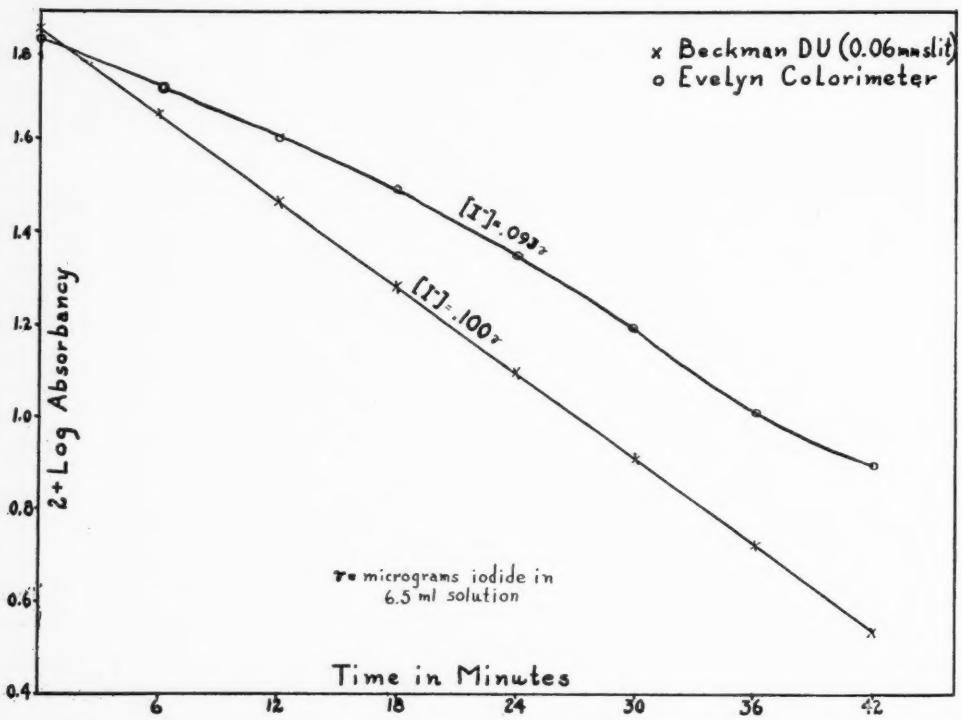

Fig. 3.—Log absorbency versus time on Beckman DU and Evelyn colorimeter (25° C.).

However, the Evelyn colorimeter produces a faint sigmoid type curve approaching linearity (Fig. 3). Therefore, equation (2) does not aptly apply on this instrument. The validity of equation (1) can, nevertheless, be tested by substituting ceric ion concentration for absorbency in the catalytic reaction. This can be seen in Fig. 4 for several concentrations of iodide. The resulting linearity demonstrates the validity of plotting log $[Ce^{4+}]$ versus time on instruments which show deviations from Beer's law rather than log O.D. versus time.

The use of log O.D. versus time for calculations on instruments other than the Beckman DU not only has the disadvantage of slight deviations from linearity but its use also introduces complicated mathematical calculations involving logarithms or requires expensive semilog paper. Moreover, the absorbancy scales on direct-reading photometers lack the sensitivity of the transmittance scale. Barker, and associates,[4] suggested the use of per cent

transmittance as an alternate to log O.D. O'Neal and Simms[7] introduced a graph to demonstrate the extent of linearity attainable by this empirical relationship. Their range of linearity extended from 35 to 60 per cent transmittance on the Coleman Universal spectrophotometer. A similar graph, using the Evelyn photometer, is shown in Fig. 1, from which it can be seen that essential linearity is possible in the range from 22 to 75 per cent transmittance. In Fig. 5, per cent transmittance was plotted against time for several concentrations of iodide, demonstrating the linearity obtained over this comparatively large range of transmittance values.

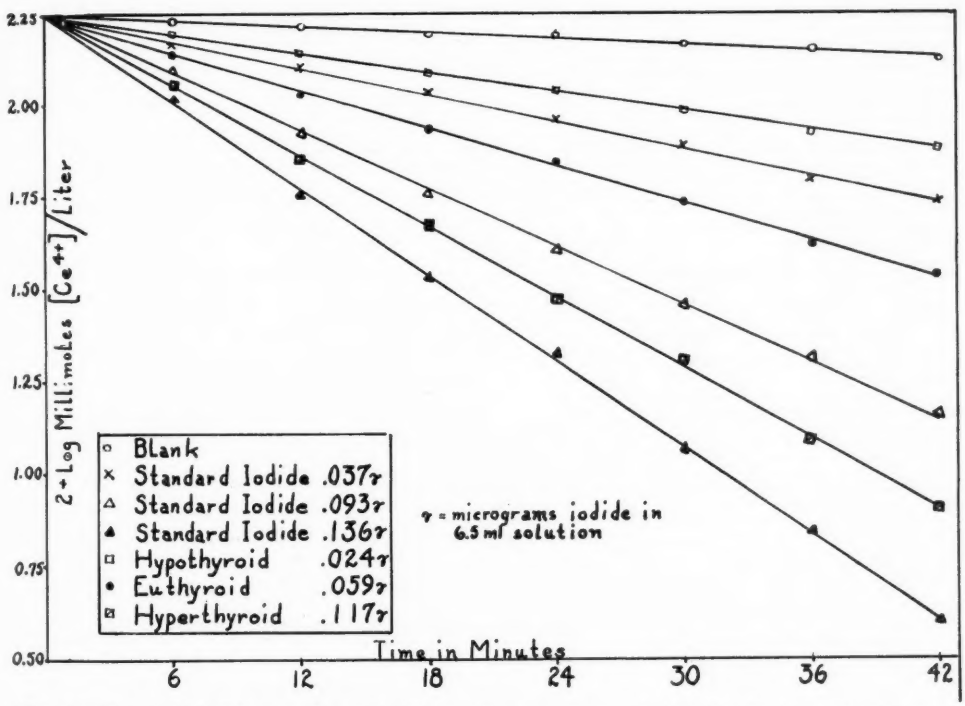

Fig. 4.—The ceric-iodide-arsenite catalytic reaction as measured with the Evelyn colorimeter on standards and samples of varied iodide concentrations (25° C.).

3. Effects of Variable Ionic Constituents.—

a. Sulphuric acid: A constant concentration of sulphuric acid associated with ceric sulfate must be maintained in the estimation of the workable concentration of ceric ion. Variable concentrations of acid in the ceric ion dilutions result in a sigmoid rather than a parabolic type curve.

The influence of the acid concentration in the reaction mixture on the light absorbancy characteristics of the ceric ion, regardless of concentration of ceric ion, was found to confirm the findings of O'Neal and Simms. Increasing concentrations of sulphuric acid to 0.175 N affected the absorption spectra considerably. Above 0.35 N, varying concentrations of acid had little or no effect. An approximately 1.15 N acid was chosen, which is well within the optimum range.

b. NaCl—Na_2SO_4 concentrations: Four milliequivalents of Na_2CO_3 was used for the digestion of protein sample because lower concentrations tended to result in some loss of iodide during the ashing process, particularly when the protein content was high. Four milliequivalents of HCl was added to the ashed sample to completely neutralize the sodium carbonate to form NaCl. With this concentration of NaCl, blank values equivalent to 1 μg, or less, of iodide were obtained. Blank values of this order do not materially shorten the range of linearity of the color reaction.

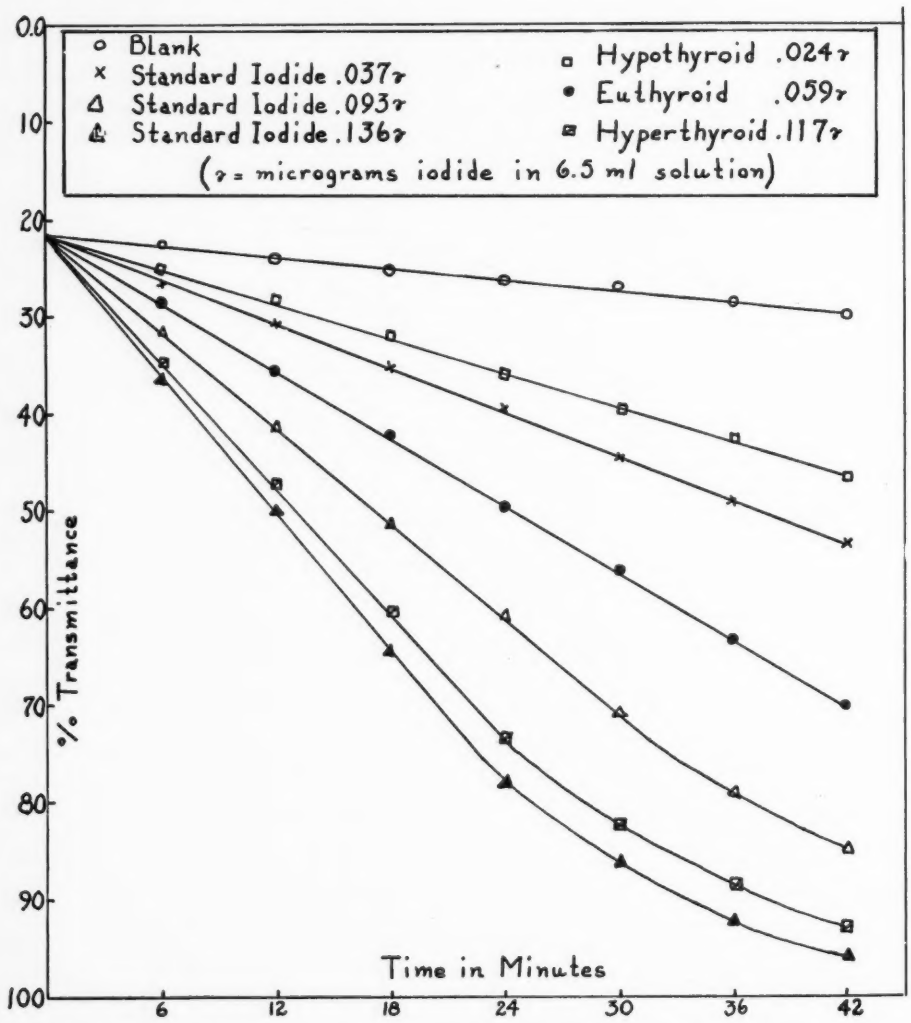

Fig. 5.—The ceric-iodide-arsenite catalytic reactions of Fig. 4 plotted in per cent transmittance versus time (25° C.).

A study of the effect of reducing the concentration of HCl, while maintaining the over-all hydrogen ion concentration by substituting H_2SO_4 to produce Na_2SO_4 from the remaining Na_2CO_3, was undertaken to determine the optimum electrolyte balance for the catalytic system. It was deemed necessary

for our purposes to first investigate the influence of varying concentrations of NaCl, in the absence of Na_2SO_4, on the rate of reaction. In Fig. 6 (solid line), the reaction rate was plotted against concentration of NaCl in milliequivalents. An increase in reaction rate was found to occur as the concentration of NaCl approached *0.7* meq., but beyond this concentration, there appeared to be little or no effect. This seems to indicate that I^+ Cl^- is responsible for the speed of the catalytic reaction.

In Fig. 6 (dashed line) is shown the result of using 4 meq. of Na_2CO_3 and reducing the amounts of HCl added; the remainder of the carbonate being neutralized by H_2SO_4. It can be seen that the reaction rate increases rapidly as the concentration of Na_2SO_4 decreases. When the Na_2CO_3 is completely neutralized with 4 meq. of H_2SO_4, the reaction rate is approximately 35 per cent of that obtained when 4 meq. of HCl is used.

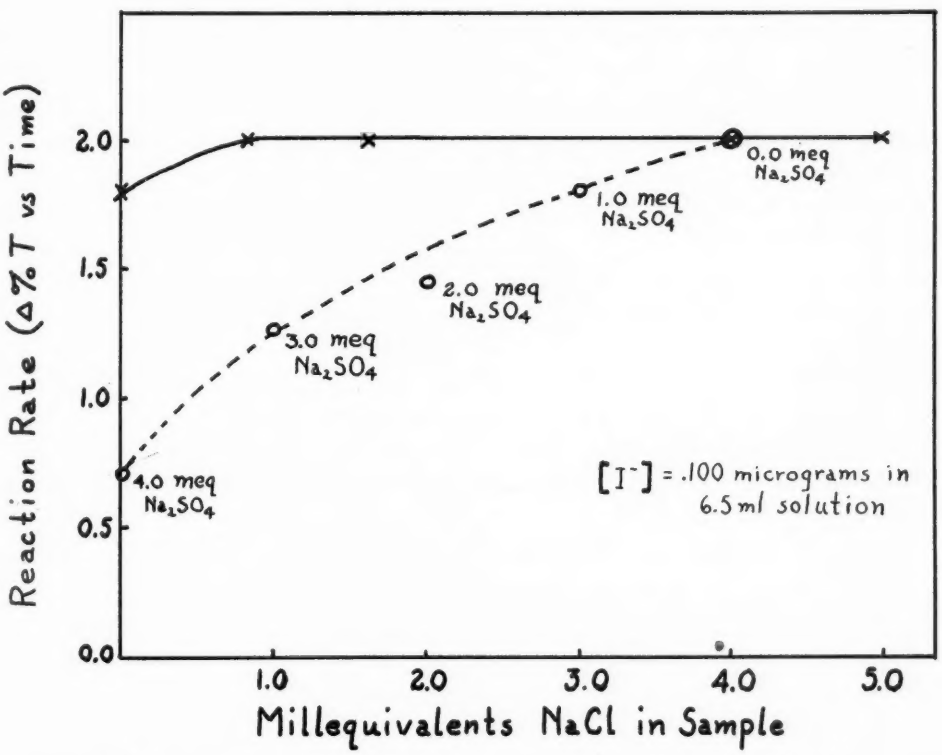

Fig. 6.—Effect of NaCl (and Na_2SO_4) concentration on reaction rate (25° C.).

It is apparent from this study that Na_2SO_4 inhibits the reaction rate. Therefore, significant errors may occur when the carbonate is not completely neutralized by the HCl. Excessive quantities of HCl must be avoided, however, in order to maintain a good range of linearity, because the trace impurities usually found in this reagent tend to produce high blanks.

c. Arsenious acid/ceric sulfate ratio: O'Neal and Simms found that the optimum equivalent ratio of arsenite ion/ceric ion in the wet-digestion method was 10:1. They also found that at lower ratios the reaction rate was affected

by slight variations in the concentration of arsenite ion, while at higher ratios these slight variations resulted in little or no change in rate of reaction. In the ashing technique presented, a ratio of 5:1 was selected, although it has been found that ratios as low as 3:1 were also satisfactory.

d. Effect of miscellaneous contaminants: Sandell and Kolthoff[9] suggested the use of an internal standard to overcome the effects of contaminating salts. It has been observed that extraneous salts introduced as a consequence of dry-ashing does not materially affect the reaction rate. By dispensing with the internal standard, as prescribed by Barker and associates a quantity of sample equivalent to 5/6 ml. of serum, rather than 3/7 ml., is used in the colorimetric procedure. In this way, the factor and consequently the error is reduced. An internal standard may be included in one of the samples, as prescribed in the procedure, to detect presence of contaminants in the sample itself.

e. Effect of iodide: The reaction between arsenite and ceric ions occurs slowly, but a small amount of iodide acts catalytically to speed up the reaction. The observed catalytic activity of iodide, in terms of $\triangle$ per cent T (or $\triangle$ log O.D.), must be directly proportional to its concentration to be useful for PBI determinations.

The linearity of the change in per cent T versus time within the limits of 22 to 75 per cent T for the Evelyn colorimeter may be expressed in terms of the slope of the line:

(1) $\triangle$ per cent T $= m \times t$

$\triangle$ per cent T = change in per cent T from zero time to time t.

m = slope of line.

Rearranging and solving for m:

$$(2)\quad m = \frac{\triangle \text{ per cent T}}{t}$$

The effect of iodide concentration, $[I^-]$, may be introduced by assuming that $[I^-]$ is proportional to slope:

$$[I^-] = f \times m = f \times \frac{\triangle \text{ per cent T}}{t}, \text{ f being a constant.}$$

If t is held constant for any concentration of iodide, then f/t is a constant, or $[I^-] = F \times \triangle$ per cent T; F is the new constant.

Transposing,

$$(3)\quad F = \frac{[I^-]}{\triangle \text{ per cent T}}.$$

This relationship is borne out by the data in Table I, columns 1-3. In column 4 is given the calculated iodide concentration based upon the factor F, obtained from a single standard solution of 0.047 μg iodide. Column 5 indicates the per cent deviation from the actual values given in column 1 if this factor, determined from a single standard, were used for calculating iodide in sample in lieu of a calibration curve. The average difference between the actual and estimated values of iodide is 1.2 per cent. The factor F is fairly constant under the same conditions of time and temperature but varies with the quality of reagents used. F varies inversely with temperature.

TABLE I. RELATIONSHIP OF IODIDE CONCENTRATION TO REACTION RATE (IN THE RANGE OF 22-75 PER CENT TRANSMITTANCE AT 25° C.)

CONCENTRATION OF IODIDE (IN μG) (1)	△ PER CENT T* (2)	F × 10^{-4}† (3)	F × PER CENT T (F = 56 × 10^{-4}) (4)	PER CENT DIFFERENCE (COLUMN 1—COLUMN 4) (5)
0.0,240	4.2	57.1	0.0,235	-2.1
0.0,370	6.6	56.1	0.0,370	0.0
0.0,470	8.4	56.0	0.0,470	0.0
0.0,930	17.2	54.1	0.0,946	+1.7
0.1,120	20.6	54.3	0.1,137	+1.5
0.1,360	26.0	54.4	0.1,383	+1.7
0.2,400	43.8	54.8	0.2,442	+1.7
			Average difference = 1.2 per cent	

*Time held constant at 12 minutes (average of readings taken at 6 and 12 minutes) and corrected for blank.

†F × 10^{-4} (constant) = $\frac{\text{Concentration of iodide}}{\triangle\ \%\ T}$.

SECTION III

CONTROL SERUM

A sample which approximately simulates the chemical composition and physical characteristics of the specimen being analyzed and contains a known concentration of PBI is the best means of controlling the accuracy and reproducibility of the procedure.

The control sera were pooled human serum which was subsequently processed to provide this laboratory with three sets of sera having concentrations covering the normal range of the test. A batch of pooled serum was filtered through a Seitz filter into a flask containing sufficient sodium Merthiolate to produce a concentration in the serum of 1:5,000. About two-thirds of the sterile serum was allowed to freeze at –10° C. in a 2-liter sterile separatory funnel. The remaining one-third of serum was tubed and stored at –10° C. and provided control serum with a normal concentration of PBI (about 5.2 μg PBI per 100 ml. serum). The funnel was removed from the freezer and the serum was allowed to thaw at room temperature in an upright position, without shaking. The lower half of this thawed serum was concentrated in respect to protein and PBI, while the upper half was low in protein and PBI. These may be separated by draining the lower half into another sterile container. Each was well shaken, and then tubed and stored at –10° C. In our experience, we were able in this way to obtain a high control serum of about 7.5 μg PBI per 100 ml. and a low of about 2.8 μg per 100 ml. serum. These sera were run along with each series of samples to be analyzed. A sufficient batch of sera was prepared to last about three to four months.

Standardization of control sera was accomplished by preparing new control sera well in advance so that they may be run in parallel with the sera already in use. Values obtained on control sera were also corroborated by other laboratories. By performing PBI analyses on specimens taken from technicians in our own laboratory and, whenever possible, by correlating our

results on patients with the physicians' diagnoses, we have found that the normal range is similar to that given by Barker and associates, that is, from 3.5 to 8.0 with a mean of approximately 5.8 μg PBI per 100 ml. serum.

Reproducibility was established by statistical analyses of data collected on the control sera over a period of six weeks. These were performed in triplicate with an average deviation of ± 5.3 per cent. The mean values of the triplicate determinations were used to estimate the reproducibility of the method in this laboratory in terms of standard deviation. The coefficient of variation was ± 3.3 per cent, and the allowable error was ± 10 per cent for three standard deviations.

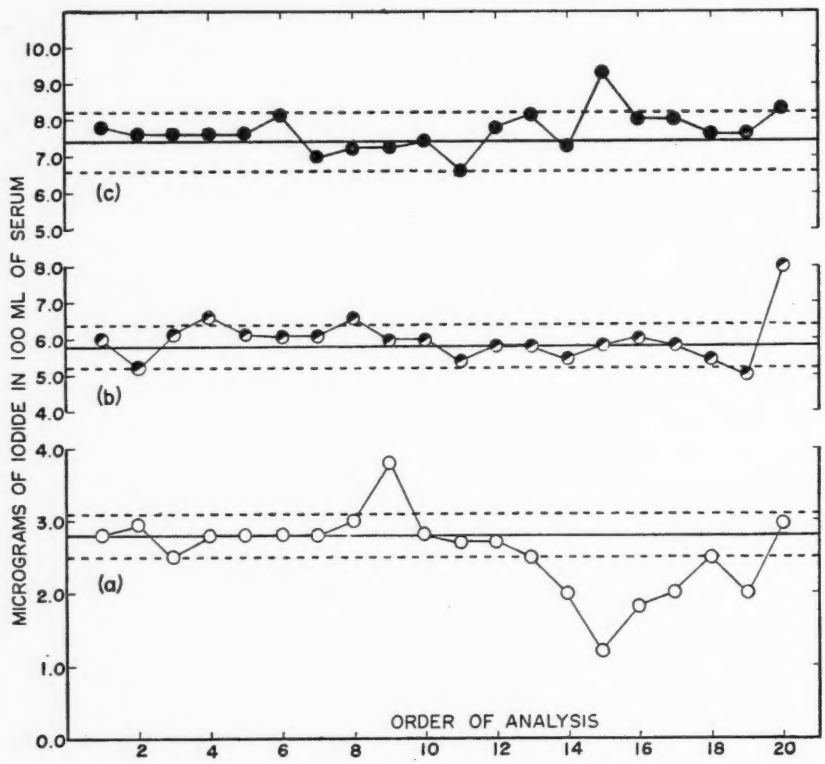

Fig. 7.—Control charts of standardized sera in micrograms per 100 ml. serum; (a) 2.8 ± 0.3, (b) 5.8 ± 0.6, (c) 7.4 ± 0.8.

Control charts were prepared with the true mean (obtained by standardization) as the ordinate and with parallel lines on either side to designate the control limits of ± 10 per cent, as calculated from the predetermined values (see Fig. 7). The order of analysis was plotted as abscissa. The control sera were run along, in triplicate, with each series of unknown samples and its mean plotted in proper chronological order.

During the thirteenth to seventeenth analyses, the low values in curve (a) were out of control. This apparent loss of iodide was traced to the ashing process, when the heating was either prolonged or allowed to reach excessive

temperatures. Since this serum has a low protein content, it points out the need for a minimal amount of protein to retain the iodide during ignition. This suggests the addition of an organic substance as an ash aid. Attempts will be made to reduce the possibility of loss of iodide in low-protein samples by the addition of a solution of pure albumin. The results of this study will be a subject of another paper.

SUMMARY

A dry-ashing technique for serum protein-bound iodine, PBI, has been presented in order to attain greater sensitivity, accuracy, and reproducibility. The decolorization of ceric ion in the catalytic reaction has been shown to be first-order. To utilize this concept of first-order in the calculations, a linear relationship must exist between concentration of ceric ion and absorbence on the photometer used in the analysis. Data have been furnished to show the extent of adherence of ceric ion to Beer's law on the Coleman 6 and 6A, the Evelyn, the Klett-Summerson, and the Beckman DU. Any instrument may be used for PBI determinations, providing a proper range has been ascertained by estimating the optimum concentration of ceric ion for the ceric-iodide-arsenite system. The use of per cent transmittance, rather than log O.D., is shown to have the advantage of simplicity. Finally, a method of quality control has been presented as a means of keeping a continuous check on the accuracy and reproducibility of the values obtained.

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REFERENCES

1. Benenson, A S., Thompson, H. L., and Klugerman, M. R.: Application of Laboratory Controls in Clinical Chemistry, Am. J. Clin. Path. **25**: 575-583, 1955.
2. Chaney, A. L.: Improvements in Determination of Iodine in Blood, Indust. & Engin. Chem., Anal. Ed. **12**: 179-181, 1940.
3. Zak, B., Willard, H. H., Myers, G. B., and Boyle, A. J.: Chloric Acid Method for Determination of Protein-bound Iodine, Anal. Chem. **24**: 1345-1348, 1952.
4. Barker, S. B., Humphrey, M. J., and Soley, M. H.: The Clinical Determination of Protein-bound Iodine, J. Clin. Invest. **30**: 55-62, 1951.
5. Van Zyl, A.: A Critical Assessment of the Distillation Technique for the Estimation of Protein-bound Iodine With Suggested Improvements, S. Afr. J. Med. Sci. **18**: 61-78, 1953.
6. Moran, J. J.: Factors Affecting the Determination of Protein-bound Iodine in Serum, Anal. Chem. **24**: 378, 1952.
7. O'Neal, L. W., and Simms, E. S.: Determination of Protein-bound Iodine in Plasma or Serum, Am. J. Clin. Path. **23**: 493-505, 1953.
8. Zieve, L., Dahle, M., and Schultz, A. L.: Comparison of Incineration and Chloric Acid Methods for Determination of Chemical Protein-bound Iodine, J. LAB. & CLIN. MED. **44**: 374-377, 1954.
9. Sandell, E. B., and Kolthoff, I. M.: Microdetermination of Iodine by a Catalytic Method, Microchimica **1**: 9-25, 1937.
10. Salter, W. T., and McKay, E. A.: Iodine in Blood and Thyroid of Man and Small Animals, J. Clin. Endocrinol. & Metab. **35**: 380-390, 1944.
11. Caraway, Wendell T.: Calculation of Protein-bound Iodine From Kinetic Data, J. Clin. Endocrinol. **12**: 1215-1219, 1952.
12. Klugerman, Maxwell R.: A Simple and Rapid Calculation in Barker's Method for Blood Protein-bound Iodine, Am. J. Clin. Path. **24**: 490-495, 1954.

QUANTITATIVE FILTER PAPER ELECTROPHORESIS OF SERUM: AUTOMATIC SCANNING AND INTEGRATION OF THE DYE-STAINED STRIP

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QUANTITATIVE analysis of dyed filter paper electropherograms of human serum has been facilitated by recent advances in instrumentation. Earlier methods based on dye elution from sections of the paper strip[1] were succeeded by direct photometric quantitation of the resolved serum electropherogram.[2, 3] Instruments have now been designed which scan the paper strip directly and automatically record the electrophoretic pattern.[4, 5]

An attempt has been made to assess the reproducibility of paper electrophoretic analysis of serum using a commercially available instrument*[6, 7] which scans the strip, records and draws the pattern, and measures the area under each peak in one combined operation, thus eliminating the time-consuming procedures of curve plotting and planimetry. This instrument has been evaluated with two dyes in common use, and the analytical accuracy has been compared with that obtained with a simpler type of nonautomatic photometer.†

METHODS

The "Analytrol" is a double cell photometer in which light transmitted through the stained strip via a narrow slit is registered by a photocell; the same light passes through another aperture to fall upon the second balancing photocell, which is partially occluded by a rotating cam. A Servo motor drives the cam so as to vary the second photocell aperture and bring the light intensity difference on the two photocells to zero. Movements of the cam are transmitted to a recording pen, which traces the pattern. An inbuilt mechanical integrator measures the areas under the peaks of the curve.

A description of the type of paper electrophoretic apparatus and procedures used and of the techniques of free electrophoresis has been previously published.[3] More recent experience indicates that superior resolutions on paper are obtainable with the Durrum-type hanging strip electrophoresis apparatus designed by Williams.[7, 8] Whatman 3 mm. filter paper was used in all studies in which bromphenol-blue dye was employed.

Two staining procedures were employed: (a) amido-black dye, for ten minutes, with decolorization by methanol acetic acid washes[3]; (b) bromphenol-blue dye,[7] for six hours, followed by two successive rinses (5 per cent acetic acid) for six minutes, fixation (0.3 per cent sodium acetate in 5 per cent acetic acid) for six minutes, and oven drying (120° C.) for at least ten minutes. Quantitation of protein concentration on the elecropherogram was performed (a) by using a nonautomatic white-light photometer ("Elphor")[2, 3]; the paper

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*"Analytrol," Spinco Model R, Spinco Division, Beckman Instruments, Inc., Belmont, Calif.

†"Elphor" photometer, Bender and Hobein, Munich, Germany.

strip, rendered translucent by oiling, was passed across a 1 ml. light slit, the optical densities read from the instrument were plotted, and the resulting curve was analyzed by planimetry; (b) by use of the "Analytrol" automatic scanner and integrator[6, 7]; strips were not oiled prior to scanning.*

RESULTS

Replicate (30) analyses of a single normal serum specimen (bromphenol-blue dye, "Analytrol" scanner), carried out under ordinary laboratory conditions by three different individuals, are presented as a basis for expected ranges of variations under normal working conditions (Table I). The standard deviations from the mean (in terms of total protein) were, for albumin, 4.5 per cent, alpha$_1$ globulin, 0.7 per cent, alpha$_2$ globulin, 1.3 per cent, beta globulin, 1.7 per cent, and gamma globulin, 2.2 per cent.† It is noteworthy that standard deviations are smaller when computed from analyses (electrophoresis and subsequent staining) of 8 strips treated simultaneously than when computed from multiple analyses at differing times (Table I).

Replicate (20) analyses of a single stained strip showed a high degree of reproducibility (Table I), suggesting that variations in analyses are due to factors inherent in the electrophoretic and staining procedures, rather than in instrumental recording of the final pattern.

It was found, however, that whereas a given scanner yields highly reproducible results with a single electropherogram, duplicate scans of the same strip by a second "Analytrol" occasionally yielded differences (in per cent of total protein) of over 4 per cent for albumin and 2 per cent for globulins. When two series of eight strips were both analyzed and scanned in different laboratories, the differences in the mean values for the various fractions were: albumin, 3.8 per cent, alpha$_1$ globulin, 0.8 per cent, alpha$_2$ globulin, 1.9 per cent, beta globulin, 2.3 per cent and gamma globulin, 0.5 per cent. Analysis of a single abnormal serum (amido-black stain) using both the "Analytrol" scanner and a nonautomatic photometer ("Elphor") did not reveal any gross differences in the performance of the two instrument (Table I).

The results of quantitative analysis by paper electrophoresis of 20 normal and miscellaneous abnormal sera, using both the "Analytrol" scanner and a nonautomatic photometer, were compared with each other and with results obtained by free electrophoresis (Table II). In most but not all instances, agreement was fairly close with all three methods, considering the standard deviations of the techniques involved and the limitations of moving boundary comparison. When deviation from free electrophoresis was considerable, particularly with sera 10, 12, 20 (all high in lipoprotein content), results by the two paper scanning techniques usually agreed well with each other. No quantitative superiority of either scanner was demonstrated on the basis of this type of comparison.

*For quantitation of amido-black stained strips analyzed on Whatman No. 1 filter paper by the "Analytrol" scanner, with cam calibrated for bromphenol-blue, an empirically derived correction factor of 1.3 was applied to all the globulin readings.

†Initial experience indicates that values (expressed as percentages of total protein) for components of normal serum, obtained with the "Analytrol" scanner, fall about means previously reported[3] for paper electrophoresis with direct scanning (albumin 57 per cent, alpha$_1$ globulin 4 per cent, alpha$_2$ globulin 9 per cent, beta globulin 14 per cent, gamma globulin 16 per cent).

TABLE I. RESULTS OF MULTIPLE PAPER ELECTROPHORETIC ANALYSES OF SERUM

ANALYSIS	DYE AND SCANNER EMPLOYED	MEANS AND STANDARD DEVIATIONS OF SERUM COMPONENTS EXPRESSED AS PERCENTAGE OF TOTAL PROTEIN GLOBULINS									
		ALBUMIN		α_1		α_2		β		γ	
		MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
Replicate (20) analyses of single electropherogram	Bromphenol-blue "Analytrol"	54.2	0.7	3.5	0.2	8.8	0.3	14.5	0.4	19.0	0.5
Replicate (8) analyses of single normal serum (strips analyzed simultaneously)	Bromphenol-blue "Analytrol"	60.4	3.6	3.3	0.5	7.2	0.9	11.4	0.6	17.7	1.7
Replicate (30) analyses of single normal serum	Bromphenol-blue "Analytrol"	59.4	4.5	3.3	0.7	7.8	1.3	12.9	1.7	16.5	2.2
	Amido-black "Analytrol"	37.6	1.2	3.0	0.6	10.5	1.8	13.6	2.0	35.3	2.1
Replicate (8) analyses of a single abnormal serum	Amido-black "Elphor"	37.3	1.2	5.6	0.5	13.5	0.8	11.4	0.6	32.3	1.7
	Free electrophoresis (single analysis)	36.7		5.1		10.2		14.6		33.4	

DISCUSSION

These results (Table I), obtained in the course of routine analyses of human sera, illustrate the degree of reproducibility of the method under average laboratory circumstances, standard deviations being somewhat greater than those obtained under more rigidly controlled conditions. The data presented (Table II) suggest that quantitative results using the "Analytrol" scanner are in agreement with those obtained with a nonautomatic photometer and also compare fairly well with free electrophoretic values, conceding the limitations of the latter for comparative purposes. Although these data, and those of others,[9-11] do draw attention to the quantitative limitations of filter paper electrophoresis of serum, it is considered that the technique and instrument under consideration are sufficiently accurate for clinical laboratory purposes.

It has been shown that variable quantitative results are due to inherent errors and factors (reviewed by Block, Durrum, and Zweig[7]) in the paper electrophoretic and dying procedures, rather than in the analyses of the final patterns. Certain of these inherent errors may be minimized by careful attention to technical detail.[7, 8] Albumin tailing, differential dye-binding of albumins and globulins,* nonstaining of lipid components of lipoprotein complexes, and nonvalidity of Beer's law† represent problems peculiar to the paper electrophoretic technique; however, the good general agreement between results with moving boundary and paper electrophoresis indicates that the sum effect of these various sources of difference is minimal.[3, 5, 13]

*An attempt was made to show that the greater dye-binding capacity of albumin than gamma globulin apparently did not apply in normal whole serum analysis (ref. 3, Table IV). However the experiment, as conducted, could not distinguish, within the limits of experimental error, whether a gamma globulin correction factor was applicable or not.[12]

†Previous studies showed that optical densities of dyed protein on a paper strip as measured by light absorption photometers obey Beer's law only within a narrow range of protein concentration[3]; however, this source of error has been obviated by incorporating optical density corrections directly into the "Analytrol" cam.

TABLE II. PAPER ELECTROPHORETIC ANALYSIS WITH QUANTITATION BY "ANALYTROL" SCANNER (A), "ELPHOR" PHOTOMETER (B), AND COMPARISON WITH FREE ELECTROPHORETIC ANALYSES (F.E.)
(Serum Components Expressed as Percentages of Total Protein)

CASE NO.	DIAGNOSIS	ALBUMIN			GLOBULINS											
					α_1			α_2			β			γ		
		F.E.*	A†	B‡	F.E.	A	B	F.E.	A	B	F.E.	A	B	F.E.	A	B
1		53.2	52.9	57.4	4.7	1.9	3.6	9.0	8.7	8.0	16.7	19.7	14.5	16.4	16.8	16.5
2		57.4	53.4	52.1	5.0	5.3	4.9	8.5	11.5	11.2	15.2	15.6	16.1	13.9	14.2	15.7
3	Normal	57.1	48.5	51.5	5.3	4.3	3.8	7.5	11.1	9.4	14.9	13.7	13.8	15.2	22.4	21.5
4		54.7	57.4	58.7	4.7	3.3	5.3	8.8	10.2	9.2	18.0	16.0	12.1	13.8	13.1	14.7
5		56.1	66.5	60.5	5.9	0.8	2.4	7.6	3.8	7.8	14.8	14.7	13.7	15.6	14.1	15.6
6		22.6	23.8	26.3	3.7	1.5	3.2	5.6	3.1	5.3	11.3	8.5	9.4	56.8	63.1	55.8
7	Portal cirrhosis	34.0	36.6	34.9	2.1	1.8	4.3	5.3	5.9	7.6	14.5	5.9	7.6	44.1	49.8	45.6
8			42.1	40.0		4.1	6.8		5.6	7.7		12.7	13.1		35.5	32.4
9			44.2	44.6		1.3	2.7		3.8	6.4		11.8	10.8		38.9	35.5
10	Biliary cirrhosis	36.6	45.6	41.8	4.7	6.9	6.0	8.2	6.3	8.2	16.3	10.5	10.5	34.2	30.7	33.3
11	Nephrotic syndrome	30.6	25.8	32.1	9.8	9.6	6.6	20.8	21.6	18.9	19.3	21.6	17.3	19.5	21.6	25.0
12		30.0	39.6	38.7	16.3	11.4	13.3	19.0	11.4	17.3	18.4	15.7	15.9	14.3	12.9	14.8
13	Multiple myeloma	45.4	38.2	46.4	9.6	9.6	7.7	14.9	15.5	12.6	18.5	22.0	16.9	11.6	14.7	16.4
14	Staphylococcal infections	53.7	55.7	53.0	6.4	7.3	7.5	12.1	10.8	11.4	14.9	13.0	12.3	12.9	13.3	15.8
15	Lymphoma	32.4	30.3	31.1	4.7	4.1	6.0	6.9	5.3	7.8	13.8	5.3	9.0	42.1	54.9	46.1
16	Pulmonary abscess	33.0	36.1	40.4	6.6	4.6	5.5	12.2	10.8	11.6	18.7	17.5	14.2	29.5	31.0	28.3
17	Recurrent pulmonary infections	53.6	59.8	51.3	7.6	4.8	5.8	13.5	13.3	15.0	13.9	10.9	11.8	11.4	11.2	16.1
18	Tuberculous adenitis	36.7	37.6	37.3	5.1	3.0	5.6	10.2	10.5	13.4	14.6	13.6	11.4	33.4	35.3	32.3
19	Bronchial asthma	55.2	55.9	47.5	4.0	3.8	5.5	9.6	9.3	9.5	15.6	11.5	13.3	15.6	19.5	24.1
20	Hypercholesterolemic xanthomatosis	46.0	60.7	60.4	5.5	2.1	2.7	10.2	6.8	7.4	27.7	15.2	14.3	10.6	15.2	15.1

*F.E.—Free electrophoretic analysis.
†A—Paper electrophoretic analysis—"Analytrol" scanner.
‡B—Paper electrophoretic analysis—"Elphor" scanner.

There is nothing in these data to suggest the superiority of one dye over another. Amido-black is more stable and requires a shorter dying time. However, quantitative results with bromphenol-blue are equally good, and since the "Analytrol" cam is calibrated for this dye, it seems to be the reagent of choice for use with this instrument.

At the present time, the best cam available for use with the "Analytrol" instrument requires the use of bromphenol-blue dye and specified conditions of electrophoresis.[7] If other dyes, filter paper, or electrophoretic conditions are preferred, a differently calibrated cam is required. Alternatively, as was done in this study, amido-black stained strips may be quantitated, using the bromphenol-blue cam, by means of a correction factor empirically derived. A universal cam, applicable for a variety of dyes, is under investigation at present.

SUMMARY

1. Reproducibility of paper electrophoretic analysis of serum has been investigated using a commercially available ("Analytrol") scanner and integrator for quantitating the stained strip.

2. Normal values and standard deviations using this scanner are presented.

3. Results obtained with the "Analytrol" were comparable with those obtained using a simpler photometer; both sets of data compared reasonably well with moving boundary analyses.

4. This scanner is an accurate, time-saving device for simple rapid analysis of paper electrophoretic strips; it does not correct the errors inherent in the paper electrophoretic procedure.

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REFERENCES

1. Cremer, H. D., and Tiselius, A.: Electrophorese von Eiweiss in Filtrierpapier, Biochem. Ztschr. **320**: 273-283, 1950.
2. Grassmann, W., and Hannig, K.: Ein quantitatives Verfahren zur Analyse der Serum proteine durch Papierelektrophorese, Hoppe Seyler's Ztschr. f. physiol. Chem. **290**: 1-27, 1952.
3. Mackay, I. R., Volwiler, W., and Goldsworthy, P. D.: Paper Electrophoresis of Serum Proteins: Photometric Quantitation and Comparison With Free Electrophoresis, J. Clin. Invest. **33**: 855-866, 1954.
4. Latner, A. L., Molyneux, L., and Rose, J. D.: A Semiautomatic Recording Densitometer for Use After Paper-strip Electrophoresis, J. LAB. & CLIN. MED. **43**: 157-164, 1954.
5. Cooper, G. R., and Mandel, E. E.: Paper Electrophoresis With Automatic Scanning and Recording, J. LAB. & CLIN. MED. **44**: 636-643, 1954.
6. Operating Instructions: Spinco Model R Analytrol. Interim edition, edited by Spinco Division, Beckman Instruments, Inc., Belmont, Calif.
7. Block, R. J., Durrum, E. L., and Zweig, G.: A Manual of Paper Chromatography and Paper Electrophoresis. Part II. New York, Academic Press Inc.
8. Williams, F. G., Jr., Pickels, E. G., and Durrum, E. L.: Improved Hanging-strip Paper-electrophoresis Technique, Science, **121**: 829-830, 1955.
9. Martin, N. H., and Franglen, G. T.: The Use and Limitations of Filters Paper Electrophoresis, J. Clin. Path. **7**: 87-105, 1954.
10. Discombe, G., Jones, R. F., and Winstanely, D. P.: The Estimation of Gamma Globulin, J. Clin. Path. **7**: 106-109, 1954.
11. Schulz, D. M., and Holdcraft, M. A.: Quantitative Paper-strip Electrophoresis, Lab. Invest. **4**: 262-274, 1955.
12. Levy, A. L., Personal communication, 1955.
13. Mackay, I. R., and Volwiler, W.: Quantitative Aspects of Filter Paper Electrophoresis of Serum, Australasian Annals of Medicine **3**: 263-269, 1954.